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(54) Title: IMPROVED TYPE III BACTERIAL STRAINS FOR USE IN MEDICINE

(57) Abstract: The present invention relates to a safe non-virulent Yersinia enterocolitica mutant strain for delivering heterologous proteins in target cells carrying mutations in at least one of the effector genes yopH, yopO, yopP, yopE, yopM, yopT genes and at least one additional mutation in the invasin genes chosen from yadA and/or inv. The present invention also relates to a safe non-virulent Yersinia enterocolitica mutant strain for delivering heterologous proteins in target cells according to claim 1 carrying mutations in all effector genes yopH, yopO, yopP, yopE, yopM, yopT genes and at least one additional mutation in the invasin genes chosen from yadA and/or inv. The present invention also relates to an expression vector for delivering a heterologous protein into a target cell using a Yersinia enterocolitica mutant strain according to any of the claims 1 to 4, which comprises in the 5' to 3' direction :(a) a promoter of a Yersinia virulon gene, (b) a first DNA sequence encoding a delivery signal from a Yersinia effector protein, operably linked to said promoter; and, (c) a second DNA sequence coding for said heterologous protein, fused in frame to the 3' end of said first DNA sequence. The present invention further relates to methods and compositions comprising (the use of) the afore-mentioned mutant strains and expression vectors.

Improved type III bacterial strains for use in medicine.

Technical field

The present invention relates to optimised bacteria in particular to improved type III strains for use in medicine. The present invention further relates to a method for classifying type-III bacteria.

Background art

For a rather long period of time, it was assumed that Gram-negative bacteria do not secrete proteins into their environment. It has been shown later on that Gram-negative bacteria can transfer proteins across their sophisticated outer membrane using specialized systems. One of these systems, type III, is a sophisticated apparatus that couples secretion with pathogenesis.

The type III system of *Yersinia* (Yop) is considered to be the archetype of a whole family of systems encountered in several gram-negative animal and plant pathogens. Other pathogenic bacteria sharing very similar type III systems are: *Pseudomonas aeroginosa*, *Bordetella pertussis* (whooping cough), *Salmonella*, *Shigella*, *enteropathogenic E.coli*, *Burholderia cepacia* and *Chlamydia*.

The three *Yersinia* species that are pathogenic to man, *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*, all share in common the ability to deliver bacterial effector proteins called "Yops" inside eukaryotic cells. *Y. enterocolitica* is a common contaminant of pork meat and an agent of mild diarrhea and abdominal pain and is frequently used to study the type III machinery. The Yop effectors allow the bacteria to survive and proliferate extracellularly in the gut-associated lymphoid system of the host. The Yop effector proteins and their machinery for export from the bacteria and transport into the eukaryotic cell are all encoded by a 70 kb virulence plasmid - which is called pYV in *Y. enterocolitica*. This whole system, along with its regulatory circuit, is called the Yop virulon (reviewed in Cornelis 2000).

The Yop virulon is a sophisticated pathogenicity system used by bacteria from the genus *Yersinia* (*Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis*) to defeat the immune system of their host. *Yersinia* are not detected inside the inflammatory or parenchymal cells of the infected animals and have the capacity to enter certain cultured epithelial cells, a process generally referred to as an invasion. This system allows extracellular bacteria adhering at the surface of eukaryotic cells to inject bacterial

effector proteins into the cytosol of these cells in order to disarm them or disrupt their communications.

The *Yersinia* virulon is composed of the following elements: 1/ a contact or type III secretion system (Yst) which is devoted to the secretion of Yop proteins out of the bacterial cells; 2/ a set of "translocators" for translocating the effector proteins into the eukaryotic host (YopB, YopD, LcrV and LcrG) 3/ a control element and recognition system (YopN and LcrG); and (iv) a set of "effector proteins" including YopE, YopH, YopO/YpkA, YopM, YopT and YopP/YopJ, which are injected (or translocated) into the eukaryotic host cells and disrupt the functions of such host cells.

Transcription of these genes is controlled both by temperature and by contact with an eukaryotic cell. When *Yersinia* is placed at 37 °C in a rich environment, the Ysc secretion apparatus is installed and a stock of Yop proteins is synthesized. As long as there is no contact with an eukaryotic cell, a stop-valve (YopN) prevents secretion. Upon contact with a target, the Ysc apparatus opens up and Yops are delivered. During their intrabacterial storage, some Yops are capped with a specific Syc chaperone carrying an anti-degradation role and regulating the hierarchy for delivering the effectors.

Six Yop effectors are known in *Yersinia* - YopE, YopH, YopM, YopT, YopO/YpkA and YopP/YopJ. The effector proteins disrupt the function of host cells in a number of ways. Out of the six proteins delivered by *Yersinia*, three (YopH, YopE, YopT) exert a negative role on cytoskeleton dynamics and contribute to the strong resistance of *Yersinia* to phagocytosis by macrophages (Cornelis and Van Gijsegem, 2000). The 23 kd YopE is a cytotoxin that disrupts the actin-microfilament structure of cultured Hela cells. The disruption of these actin filaments by YopE leads to inhibition of phagocytosis by polymorphonuclear leukocytes (PMNs) and macrophages. The 51 kd YopH is a protein tyrosine phosphatase (PTPase) related to eukaryotic PTPases, which acts on tyrosinephosphorylated proteins of infected macrophages. Dephosphorylation of these proteins leads to disruption of the signal transduction pathways involved in cellular uptake of bacteria, possibly by specifically inhibiting self-induced calcium-signaling. Presumably as a result of this action, YopH inhibits bacterial uptake and oxidative burst by cultured macrophages. In addition, YopH suppresses T- and B-lymphocyte activation via their antigen receptors. T-cells are impaired in their ability to produce cytokines and B-cells are unable to upregulate surface expression of the co-stimulatory molecule B7.2 in response to antigen activation. Cytotoxin YopT inactivates a GTPase that regulates the formation of stress fibers. YopO (or YpkA) is an 81 kd serine/threonine kinase, which

is targeted to the inner surface of the plasma membrane of the eukaryotic cell and might function to interfere with the signal transduction pathway of the eukaryotic cell. YopM is an acidic 41 kd protein having 12 leucine-rich repeats. The function of YopM is not well understood as well as the function of similar proteins from other type III containing bacteria. YopP (also called YopJ in *Y. pestis* and *Y. pseudotuberculosis*) induces apoptosis of macrophages. YopP also blocks the release of TNFα by macrophages and IL-8 by epithelial cell, which leads to a significant reduction in inflammation.

The Yop effectors require the Ysc proteins in order to cross the two bacterial membranes. The latter constitute the Ysc apparatus, which is supposed to form some kind of a needle. Four "translocators" are essential for the translocation of the effector Yops across the eukaryotic membrane: YopB, YopD, LcrV and LcrG. Two of them, YopB and YopD form a pore in the eukaryotic cell membrane.

In order for *Y.enterocolitica* to translocate Yops into eukaryotic cells the bacteria must adhere to the surface of eukaryotic cells. The attachment induces opening of the Ysc secretion channel and increased production of the Yops. The two major determinants of adhesion in *Y. enterocolitica* are Invasin and YadA. Invasin is a chromosomally encoded protein which binds β1 integrins on the surface of eukaryotic cells, while YadA is encoded by the virulence plasmid itself and binds a variety of extracellular proteins including fibronectin and collagen. There may be a receptor on the eukaryotic cell surface that interacts with a ligand on the bacterial surface and leads to a signal for Yop injection. In *Y. enterocolitica* YopN, TyeA and/or LcrG have the potential to be the bacterial ligand, as bacteria carrying mutations in any one of these genes have a deregulated Yop secretion phenotype.

Translocation of the Yop effectors into eukaryotic cells could be demonstrated using hybrid proteins fusing truncated Yop effector proteins of different length with certain reporter enzymes such as the calmodulin-activated adenylate cyclase domain (Cya) of the *Bordetella pertussis* cyclolysin. This illustrated that Yops are translocated into eukaryotic cells but also that *Yersinia* can deliver foreign proteins into eukaryotic cells (or Cya). By applying this approach, it was suggested in US5,965,381 that the N-terminal domain of YopE, YopM, YopH, YopO/YpkA, and YopP/YopJ of *Y. enterocolitica* could be used as "delivery signal" to deliver heterologous proteins in eukaryotic cells. In this patent the use of such expression vector in combination with a mutant *Yersinia* strain to deliver a desired protein into eukaryotic cells was demonstrated. This mutant *Yersinia* was defined to be deficient in the production of functional effector proteins, but

still endowed with a functional secretion and translocation system. This approach was found to be useful not only for studying the function of a given protein, but also for designing therapeutic approaches. The inventors pointed out that these mutant *Yersinia* had a diminished toxicity, i.e., toxicity which does not completely disable or kill the target cell. Nevertheless, when using recombinant strains for medical purposes it is of great importance that the strains used are safe to use. Its is also of prime importance that, especially when starting from a virulent strain, all virulent factors are deleted.

In addition, it has been recently shown that the translocation of the Yop effector proteins using *Yersinia enterocolitica* is not selective (Boyd *et al.* 2000). This would suggest that, although this system seemed to be a potential vector for delivering heterologous proteins into eukaryotic cells, the delivery is more or less random. Unfortunately, such a non-selective delivery may elicit unwanted side effects of the medicament comprising this mutant strain; this is not be permitted in medicine.

Objects of the invention

The present invention is therefore directed towards the development of an improved *Yersinia* vector strain, whereby "improved" means that the strain is much safer to use in medicine. With "safe" is meant that virulence of the strain is reduced to a minimun, creating a non-harming delivering vector; in addition, "safer" also means that the corresponding strain does not include any factor which is not allowed by the FDA. With "vector strain " is meant a cell which is used to deliver genetic information (DNA or protein) in another cell. The advantage of a bacterial vector strain versus a viral vector is that antibiotics may still be used to eliminate the vector strain in a subject when it is not needed anymore; this is not possible when using viral vectors.

A particular object of the present invention is directed towards the development of a well considered *Y.enterocolitica* which is safe to use; and in addition allowing a more specific delivery of heterologous proteins to specific target cells. A further object of the present invention is directed towards the development of a well established classification system in order to determine the prototype strain that can be used to study type III molecules/genes isolated from non-cultivable pathogenic strains.

These aims have been met by following embodiments.

Detailed description of the invention

The present invention relates to a safe non-virulent *Yersinia enterocolitica* mutant strain for delivering heterologous proteins in target cells carrying mutations in at least one of the effector genes yopH, yopO, yopP, yopE, yopM, yopT genes and at least one additional mutation in the invasin genes chosen from yadA and/or inv.

The present invention relates to the production of a disarmed vector *Yersinia* strain which is totally non-pathogenic and safe to use for medical purposes but still carrying the capacity of transferring heterologous proteins into target cells. The term "*Yersinia*" as used herein means all species of *Yersinia*, including *Yersinia enterocolitica, Yersinia pseudotuberculosis* and *Yersinia pestis*.

The Yop virulon of *Y. enterocolitica* encodes six Yop effectors - YopH, YopO/YpkA YopP/YopJ, YopE, YopM and YopT. As described above, functional effector proteins induce a range of modifications to the normal processes of the eukaryotic cells and are toxic to the target cell. A "functional effector protein" refers to an effector protein having a defined catalytic activity and which is capable of eliciting specific toxicity toward the target cells. When using *Yersinia* as a vector organism to deliver heterologous proteins in eukaryotic cells, especially for medical use, it is of primary importance that all toxic elements are eliminated. Therefore the present inventors suggest to mutate some, preferentially all the genes that code for the six effector proteins, this, in addition to the mutation of one of the invasin genes.

A preferred mutant *Yersinia* strain of the present invention is a multiple-mutant *Yersinia* strain in which all the effector-encoding genes are mutated such that the resulting *Yersinia* no longer produce any functional effector proteins, this, in addition to the mutation of one of the invasin genes. Therefore present invention also relates to a safe non-virulent *Yersinia enterocolitica* mutant strain for delivering heterologous proteins in target cells carrying mutations in all effector genes yopH, yopO, yopP, yopE, yopM, yopT genes and at least one additional mutation in the invasin genes chosen from yadA and/or inv. Such multiple-mutant *Yersinia* strain is designated as yopHOPEMTinv or yopHOPEMTyad for *Y. enterocolitica*; yopHAJEMTinv or yopHAJEMTyad for *Y. pseudotuberculosis*. With "yopHOPEMT" is meant a strain that is mutated in the effector genes YopH, YopO, YopP, YopE, YopM and YopT. Inv and yad reflects the mutations present in the Inv or YadA genes respectively.

One example of such a yopHOPEMTinv strain is *Y. enterocolitica* APB40 (pIML421). This strain was deposited at the Belgian Coordinated Collection of Microrganisms – BCCM/LMG Collection (Laboratorium for Microbiology, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium) under deposit number MLG P-21014 on 24 September 2001 by Prof. G. Cornelis (Unité de Pathologie Microbienne (MIPA), Université Catholique de Louvain, Av. Hippocrate 74, B-1200 Brussels, Belgium). Plasmid pIML421 was generated by crossing serially into the wild-type virulence plasmid pYV40 the following mutations : *yopH*Δ1-352, *yopO*Δ65-558, *yopP*23, *yopE*21, *yopM*23, *yopT*135. In these mutation , the numbers indicate the number of codons that are either left or are deleted if the number is preceeded by the Δ symbol. All these mutations have been crossed using mutator plasmids derived from the suicide vector pKNG101 (streptomycin resistant) and containing the relevant mutations. The mutators are pAB31 (*yopH*Δ1-352), pAB25 (*yopO*Δ65-558) , pMSK7 (*yopP*23), pPW52 (*yopE*21), pAB38 (*yopM*23), pIML200 (*yopT*135). To generate the *inv* mutant APB40(pIML421), the *inv* allele was disrupted on the chromosome of E40(pIML421) by insertion of the *inv* mutator pMS154. pMS154 is a derivative of our suicide plasmid pKNG160 (tetracycline resistant) in which we cloned a 1400-bp internal fragment of *inv* isolated from plasmid pINT192, received from Virginia Miller (University of California, Los Angeles).

Another example of is *Y. enterocolitica* MRS40 (pIML421). This strain was deposited at the Belgian Coordinated Collection of Microrganisms – BCCM/LMG Collection (Laboratorium for Microbiology, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium) under deposit number MLG P-21013 on 24 September 2001 by Prof. G. Cornelis (Unité de Pathologie Microbienne (MIPA), Université Catholique de Louvain, Av. Hippocrate 74, B-1200 Brussels, Belgium).

Yersinia enterocolitica strains produce an enterotoxin called Yst (Delor and Cornelis, 1990). This enterotoxin is encoded by the *yst* gene present on the chromosome. A strain producing an enterotoxin would not be acceptable for use in patients. Hence, the *yst* gene has to be removed by using the mutator pID101 which replaces the yst gene by a gene encoding the luciferase from *Vibrio harveyi* (Delor and Cornelis, 1992).

Under some circumstances, it may be desired to mutate only some but not all of the genes described here before. Indeed, if a strain is too severely disarmed, it might be eliminated from the organism before it made its effect. Accordingly, the present invention further contemplates polymutant *Yersinia* other than sixtuple-mutant *Yersinia*, e.g., double-mutant, triple-mutant, quadruple-mutant, quintuple-mutant and sixtuple-

mutant *Yersinia*. Also here the nomenclature only refers to the number of effector genes which are affected.

Possible mutation combinations are: YopHinv, YopHyad, YopHinvyad, YopOinv, YopOyad, YopOinvyad, YopPinv, YopPyad, YopPinvyad, YopEinv, YopEyad, YopEinvyad, YopMinv, YopMyad, YopMinvyad, YopTinv, YopTyad, YopTinvyad, YopHOinv, YopHOyad, YopHOinvyad, YopHPinv, YopHPyad, YopHPinvyad, YopHEinv, YopHEyad, YopHEinvyad, YopHMinv, YopHMyad, YopHMinvyad, YopHTinv, YopHTyad, YopHTinvyad, YopOPinv, YopOPyad, YopOPinvyad, YopOEinv, YopOEyad, YopOEinvyad, YopOMinv, YopOMyad, YopOMinvyad, YopOTinv, YopOTyad, YopOTinvyad, YopPEinv, YopPEyad, YopPEinvyad, YopPMinv, YopPMyad, YopPMinvyad, YopPTinv, YopPTyad, YopPTinvyad, YopEMinv, YopEMyad, YopEMinvyad, YopETinv, YopETyad, YopETinvyad, YopMTinv, YopMTyad, YopMTinvyad, YopHOPinv, YopHOPyad, YopHOPinvyad, YopHOEinv, YopHOEyad, YopHOEinvyad, YopHOMinv, YopHOMyad, YopHOMinvyad, YopHOTinv, YopHOTyad, YopHOTinvyad, YopHPEinv, YopHPEyad, YopHPEinvyad, YopHPMinv, YopHPMyad, YopHPMinvyad, YopHPTinv, YopHPTyad, YopHPTinvyad, YopHEMinv, YopHEMyad, YopHEMinvyad, YopHETinv, YopHETyad, YopHETinvyad, YopHMTinv, YopHMTyad, YopHMTinvyad, YopOPEinv, YopOPEyad, YopOPEinvyad, YopOPMinv, YopOPMyad, YopOPMinvyad, YopOPTinv, YopOPTyad, YopOPTinvyad, YopPEMinv, YopPEMyad, YopPEMinvyad, YopPETinv, YopPETyad, YopPETinvyad, YopEMTinv, YopEMTyad, YopEMTinvyad, YopOETinv, YopOETyad, YopOETinvyad, YopOEMinv, YopOEMyad, YopOEMinvyad, YopOMTinv, YopOMTyad, YopOMTinvyad, YopHOPEinv, YopHOPEyad, YopHOPEinvyad, YopOPEMinv, YopOPEMyad, YopOPEMinvyad, YopPEMTinv, YopPEMTyad, YopPEMTinvyad, YopHPEMinv, YopHPEMyad, YopHPEMinvyad, YopHEMTinv, YopHEMTyad, YopHEMTinvyad, YopOEMTinv, YopOEMTyad, YopOEMTinvyad, YopHOEMinv, YopHOEMyad, YopHOEMinvyad, YopHOETinv, YopHOETyad, YopHOETinvyad, YopHOMTinv, YopHOMTyad, YopHOMTinvyad, YopOPETinv, YopOPETyad, YopOPETinvyad, YopOPMTinv, YopOPMTyad, YopOPMTinvyad, YopHOPMinv, YopHOPMyad, YopHOPMinvyad, YopHOPTinv, YopHOPTyad, YopHOPTinvyad, YopHPETinv, YopHPETyad, YopHPETinvyad, YopHPMTinv, YopHPMTyad, YopHPMTinvyad, YopHOPEMinv, YopHOPEMyad, YopHOPEMinvyad, YopHOPETinv, YopHOPETyad, YopHOPETinvyad YopHOPMTinv, YopHOPMTyad, YopHOPMTinvyad YopHOEMTinv, YopHOEMTyad, YopHOEMTinvyad YopHPEMTinv, YopHPEMTyad, YopHPEMTinvyad YopOPEMTinv,

YopOPEMTyad, YopOPEMTinvyad, YopHOPEMTinv, YopHOPEMTyad and YopHOPEMTinvyad.

According to the present invention the mutant strain is used to deliver heterologous proteins into target cells. It was shown that deletion of translocators (YopB, YopD and LcrV) resulted in a translocation-deficient strain (Boyd *et al.* 2000). Therefore the present inventors suggest to keep these genes active. Also the control elements and the *ysc* genes which are devoted to the delivery of the Yop proteins are essential in the delivery system, these are preferentially kept functionally active in the mutant vector strain.

For the purpose of the present invention, the terms "mutant strain", "vector strain" or "mutant vector strain" refer to the strain as such carrying the different mutations as described. The term "recombinant *Yersinia*", or "recombinant strain" or "recombinant vector strain" used herein refers to the mutant *Yersinia* genetically transformed with the expression vectors of the present invention. "Eukaryotic cells" as used herein, the surface to which *Yersinia* adhere to, are also referred to as "target cells" or "target eukaryotic cells". In some cases, target cells have a more limited definition, meaning the cells that are specifically recognized by the vector cells. It is clear from the content of the sentence when this latter definition is used. With "specifically" it is implied that there is substantially no cross-interaction of the vector strains with other non-target cells. With "target cell" is meant all cells which are able to interact with type III carrying bacteria and able to accept foreign proteins from this organism.

It was shown that in order for *Yersinia* species to translocate Yops into eukaryotic cells the bacteria must adhere to the surface of eukaryotic cells. The attachment induces opening of the Ysc secretion channel and increased production of the Yops. The two major determinants of adhesion in *Y. pseudotuberculosis* and *Y. enterocolitica* are Invasin and YadA. In addition, in *Y. enterocolitica* YopN, TyeA and/or LcrG have the potential to be the bacterial ligand necessary to interact with a receptor present on the eukaryotic surface. Nevertheless, it has been recently shown that in *Y enterocolitica* even such a complex system does not allow selective translocation in target cells (Boyd *et al.* (2000)). Although it is evident that these Inv and Yad molecules are essential for the transfer of Yop effectors to target cells, the inventors suggest to tackle this problem by eliminating these genes.The inventors found that translocation can be made more specific so that only target cells (eg. phagocytic cells or cancer cells) are targeted. A first step in this approach is to eliminate the elements which trigger this aspecific interaction. The inventors proved that invasin and / or YadA, two powerful adhesins were required

for translocation of Yop into non-phagocytic cells. The inventors found that by eliminating the Inv and YadA this aspecific interaction could be abolished without interfering with the transfer capacity of this vector strain to phagocytic cells. If one targets non-phagocytic cells, it is necessary to provide Yersinia with an specific adhesin that recognizes the cells to be targetted. In addition, the inventors found that deletion of the two invasin genes (yadA and inv) avoids invasion at the level of the Peyers patches. This will limit the sphere of action of the vector to the lumen of the intestinal tractAs used herein, the "secretion" of a protein refers to the transportation of such protein outward across the cell membrane of a *Yersinia*. The "translocation" of a protein refers to the transportation of such protein across the plasma membrane of an eukaryotic cell into the cytosol of such eukaryotic cell.

The inventors found that the combination of YopH, YopE and YopT are necessary for the dynamics of phagocytosis by macrophages *in vitro*.

Six effector genes have been cloned from *Y. enterocolitica* which are YopE, YopH, YopO, YopM, YopT and YopP. The equivalent effector genes have been cloned from *Y. pseudotuberculosis* and are named as YopE, YopH, YpkA, YopM, and YopJ, respectively. Some effector genes have also been cloned from *Y. pestis*. The nucleic acid sequences of these Yop genes are available to those skilled in the art, e.g., in the Genebank Database.

For the purpose of the present invention, the effector-encoding genes are denoted by italicized letters to be distinguished from the effector proteins. Letters of lower case that are italicized denote mutant effector genes. For example, YopE refers to the effector protein encoded by the *yopE* gene. *yopE+* represents the wild type gene, while *yopE* represents a gene having a mutation.

According to the present invention, a mutant *Yersinia* strain can be generated by introducing at least one mutation into at least one effector-encoding gene, this combined with at least one mutation in one of the invasin genes. Preferably, such effector-encoding genes include YopE, YopH, YopO/YpkA, YopM, YopT and YopP/YopJ. The skilled artisan may employ any number of standard techniques to generate mutations in these Yop genes. Sambrook *et al.* describe in general such techniques. See Sambrook *et al.* (1989) Molecular Cloning: A Laboratory Manual, Second Edition Cold Spring Harbor Laboratory Press: Cold Spring Harbor, N.Y. The present invention illustrates that a safe non-virulent *Yersinia enterocolitica* mutant strain for delivering heterologous proteins in

target cells may contain (a) mutation(s) located in the non-coding sequence of said gene and/or in the coding sequence of said gene.

The term "mutation" is used herein as a general term and includes changes of both single base pair and multiple base pairs. Such mutations may include substitutions, frame-shift mutations, deletions, truncations, insertions, duplications or any modification in the nucleic acid encoding said polypeptide, or at a different location in the genome of said cells, influencing the expression of said nucleic acid or polypeptide. In case point mutations occur, the number of nucleotides will be identical compared to the original sequence; only a change in nucleotide sequence can be observed. This stands in contrast with the other listed mutations where the number of the nucleotides will be different from the number observed in the wild type sequence and consequently will also reflect in a change of the nucleotide sequence.

In accordance with the present invention, the mutation can be generated in the promoter region of an effector-encoding gene so that the expression of such effector gene is abolished.

The mutation can also be generated in the coding region of an effector-encoding gene such that the catalytic activity of the encoded effector protein is abolished. The "catalytic activity" of an effector protein refers to the anti-target cell function of an effector protein, i.e., toxicity. Such activity is governed by the catalytic motifs in the catalytic domain of an effector protein. The approaches for identifying the catalytic domain and/or the catalytic motifs of an effector protein are well within the knowledge of those skilled in the art. See, for example, Sory *et al.* (1995), Boland *et al.* (1996) and Cornelis *et al.* (1997).

Accordingly, one preferred mutation of the present invention is a deletion of the entire catalytic domain. Another preferred mutation is a frameshift mutation in an effector-encoding gene such that the catalytic domain is not present in the protein product expressed from such "frameshifted" gene. A most preferred mutation is a mutation with the deletion of the entire coding region.

Other mutations are also contemplated by the present invention, such as small deletions or base pair substitutions, which are generated in the catalytic motifs of an effector protein leading to destruction of the catalytic activity of a given effector protein.

The mutations that are generated in the Yop genes may be introduced into *Yersinia* by a number of methods. One such method involves cloning a mutated Yop gene (i.e., a yop gene), into a "suicide" vector which is capable of introducing the mutated yop

sequence into *Yersinia* via allelic exchange. Such "suicide" vectors are described by Kaniga *et al.* (1991) Gene 109: 137-141 and by Sarker *et al.* (1997) Mol. Microbiol 23: 409-411.

In this manner, mutations generated in multiple Yop genes may be introduced successively into *Yersinia*, giving rise to polymutant recombinant *Yersinia*. The order in which these mutated yop sequences are introduced is not important.

In this way, a mutant disarmed *Y.enterocolitica* strain is created which is safe to be used for medical use causing no infection in the patient.

Additional mutations can be introduced in order to guarantee that the mutant *Yersinia* vector strain is safe to use. Therefore the present invention also relates to the optional mutation onto the previously described mutation of YopR, YopQ, YlpA, YomA, Yst, β-Lac or any gene linked to the iron acquisition system.

YopR (encoded by the *yscH* gene) and YopQ (encoded by *yopQ* are transported by the type III system but no clear functions have been assigned for both proteins yet. It has been suggested for YopQ that it may play a role in regulation the size of the pore made by the type III system. The fact that both proteins are conserved within the type III system suggests their role in virulence. In addition, as both proteins use the transport system they may compete with the transport of the heterologous protein. Therefore, the present inventors suggest to mutate both yscH and *yopQ* genes (Boyd *et al.* 2000).

In the mouse model, there is no clear difference in the virulence between wild-type W22703 and an *ylpA* mutant called W22708 (pYL4). However, this observation is of little value because W22703 is of very low virulence for the mouse and hence the model does not allow to detect minor differences. The inventors have shown that the expression of lipoprotein YlpA (China *et al.*, 1990) is regulated by the same regulator that regulates *yop*, *ysc* and *yad*A genes. Thus, YlpA is expressed at the bacterial surface only at 37°C. Moreover, YlpA is conserved among all the pathogenic strains. For these reeasons, the inventors consider that this outer membrane protein must play a role in virulence.

YomA (Iriarte,Mand Cornelis,G.R., 1999) is an putative outer membrane protein with unknown function and conserved over diverse type III systems. The expression of YomA is also regulated like that of the Yops, YadA and YlpA. Moreover, it is conserved among the pathogenic strains. Because of this, the inventors suggest it must have a role in virulence. Mutants are presently beeing engineered and their phenotype will be characterized with respect to animal virulence, adherence and proteins translocation.

The present invention also suggests to delete the chromosome gene encoding the thermostable enterotoxin Yst (Delor and Cornelis, 1992). As this is a toxin it is preferably removed from the mutant vector strain.

Y. enterocolitica carries on its chromosome two endogenous β-Lactamase genes, β-lactamase A and β-l actamase B (Cornelis and Abraham, 1974). β-Lactamase A is essentially active against ampicillin while β-lactamase B is a cephalosporinase. Removal of β-LactamaseA gene makes this vector strain sensitive to the common antibiotic ampicillin and equivalent antibiotics. Especially when using a strain for medical purpose, it is of prime importance that it can be readily eliminated. Therefore, the present invention also suggests to eliminate β-Lactamase A by mutation, using the mutator plasmid pKNG105 (Kaniga, Delor and Cornelis, 1991). As an example, strain MRS40 is the *blaA* mutant of strain E40.

The present invention also suggests to devoid the mutant vector strain of any iron acquisition system. *Y. enterocolitica* is then dependent from iron supplemented in the medium. As iron is limited in body fluids, growth *in vivo* of this strain is limited. This contributes to the production of a much safer and well-considered vector strain. Strains of serotype O :9 , like E40 or W22703 described here, are naturally devoid of an iron chelating system.

The delivery vector plasmid contains the 5' end of a yop gene followed by a restriction site, so as to allow in frame cloning of genes of interest. With delivery vector, or expression vector is meant the DNA fragment that carries the heterologous gene allowing its expression in the vector strain.

A further aspect of the present invention is directed to an expression vector for use in combination with the a mutant *Yersinia* strain according to the present invention to deliver a desired protein into eukaryotic cells. In accordance with the present invention, such a vector is characterized by (in the 5' to 3' direction) a promoter, a first nucleic acid sequence encoding a delivery signal from a *Yersinia* effector protein, operably linked to said promoter, a second nucleic acid sequence fused thereto coding for a heterologous protein to be delivered.

In accordance with the present invention, the promoter of the expression vector is preferably derived from a *Yersinia* virulon gene. A "*Yersinia* virulon gene" refers to genes on the *Yersinia* pYV plasmid, the expression of which is controlled both by temperature and by contact with a target cell. See review by Cornelis *et al.* (1997). Such genes include genes coding for elements of the secretion machinery (the Ysc genes), genes

coding for translocators (YopB, YopD, and LcrV), genes coding for the control elements (YopN and LcrG), and genes coding for effectors (YopE, YopH, YopO/YpkA, YopM and YopP/YopJ).

In a preferred embodiment of the present invention, the promoter is from an effector-encoding gene selected from any one of YopE, YopH, YopO/YpkA, YopM and YopP/YopJ. More preferably, the promoter is from YopE.

Further in accordance with the present invention, a first DNA sequence coding for a delivery signal is operably linked to the promoter.

"A delivery signal", as described herein above, refers to a polypeptide which can be recognized by the secretion and translocation system of *Yersinia* and therefore directs the secretion and translocation of a protein into a target cell.

The term "delivery" used herein refers to the transportation of a protein from a *Yersinia* to a eukaryotic cell, including the steps of expressing the protein in the *Yersinia*, secreting the expressed protein(s) from such *Yersinia* and translocating the secreted protein(s) by such *Yersinia* into the cytosol of the eukaryotic cell.

According to the present invention, such a "delivery" polypeptide is derived from an effector protein. The effector proteins include YopE, YopH, YopO/YpkA, YopM, and YopP/YopJ. Preferably, the effector protein is YopE. More preferably, the effector protein is YopE of *Yersinia enterocolitica*.

One skilled in the art is familiar with the methods for identifying the polypeptide sequences of an effector protein that are capable of delivering a protein. For example, one such method is described by Sory *et al.* (1994). Briefly, polypeptide sequences from various portions of the Yop proteins can be fused in-frame to a reporter enzyme such as the calmodulin-activated adenylate cyclase domain (or Cya) of the *Bordetella pertussis* cyclolysin. Delivery of a Yop-Cya hybrid protein into the cytosol of eukaryotic cells is indicated by the appearance of cyclase activity in the infected eukaryotic cells that leads to the accumulation of cAMP. This accumulation can be measured and gives an indication upon efficiency of the delivery signal. Examples of such delivery signal polypeptides include from *Y. enterocolitica*: YopE130 (the N-terminal 130 amino acids of YopE), YopE50, YopM100 and YopH71.

By employing such an approach, one skilled in the art can determine, if desired, the minimal sequence requirement, i.e., a contiguous amino acid sequence of the shortest length, that is capable of delivering a protein. See, e.g., Sory *et al.* (1994). Accordingly, preferred delivery signals of the present invention consists of at least the minimal

sequence of amino acids of a Yop effector protein that is capable of delivering a protein. Preferably, such a fragment consists of 15 to 50, preferably 20, 25, 30, 35, 40 contiguous amino acids of a protein.

Further in accordance with the present invention, a second DNA sequence encoding a heterologous protein is fused in frame to the first DNA sequence in the instant vector for delivery into eukaryotic cells. The term "heterologous protein" used herein refers to a protein other than a *Yersinia* Yop protein. "Yop proteins" refer to *Yersinia* virulon proteins that are secreted, including the translocators and the effectors.

According to the present invention, "a heterologous protein" includes naturally occurring proteins or parts thereof. The term "part of a protein" includes a peptide or polypeptide fragment of a protein that is of sufficient length to carry an activity. Preferably, such a fragment consists of at least 8 or 9 contiguous amino acids of a protein. The present invention illustrates that said heterologous protein is a protein that is naturally occurring or a protein which is encoded by a gene but whereby the protein has never been demonstrated in nature. With "a protein which is encoded by a gene but whereby the protein has never been demonstrated in nature" is meant that a protein/peptide can be made from a synthetic gene, whereby the protein/peptide may have a structure similar or non-similar to natural proteins/peptides. Proteins/peptides which are similar to natural proteins/peptides may be derivatives or variants of natural proteins; or may be structural analogues of organic structures. These heterologous entities are condemned to be peptidic structures as they need to be expressed from DNA coding sequences. This "non-natural protein" also includes artificially engineered proteins or parts thereof, such as fusion of two or more naturally occurring proteins or parts thereof, polypeptides (in-frame fusion of two or more peptide epitopes) as exemplified by Thompson *et al.* (1995) in Proc. Natl. Acad. Sci. USA 92: 5845-5849.

The protein expressed from the fused first and second DNA sequences is also termed as a "fusion protein" or a "hybrid protein", i.e., a hybrid of *Yersinia* delivery signal and a heterologous protein.

There is no particular limitation in the heterologous protein that can be delivered. The present invention particularly contemplates proteins, such as, e.g., tumor associated proteins, antigens of pathogens, killer agents and anti-inflammatory compounds. According to the present invention, said heterologous protein, produced upon recombinant expression, is an antigen, a toxin or a drug.

In a preferred embodiment of the present invention, said heterologous protein is an antigen or at least one epitope of said protein whereby the antigen (epitope) is (is derived from) a tumor associated protein (TAA), an infection-related protein.

A "tumor associated protein" refers to a protein that is specifically expressed in tumors or expressed at an abnormal level in tumors relative to normal tissues. Such tumor associated proteins include, but are not limited to, members of the MAGE family, the BAGE family (such as BAGE-1), the DAGE/Prame family (such as DAGE-1), the GAGE family, the RAGE family (such as RAGE-1), the SMAGE family, NAG, Tyrosinase, Melan-A/MART-1, gp100, MUC-1, TAG-72, CA125, mutated proto-oncogenes such as p21ras, mutated tumor suppressor genes such as p53, tumor associated viral antigens (e.g., HPV16 E7), HOM-MEL-40, HOM-MEL-55, NY-COL-2, HOM-HD-397, HOM-RCC-1.14, HOM-HD-21, HOM-NSCLC-11, HOM-MEL-2.4, and HOM-TES-11. Members of the MAGE family include, but are not limited to, MAGE-1, MAGE-2, MAGE-11. Members of the GAGE family include, but are not limited to, GAGE-1, GAGE-6. See, e.g., review by Van den Eynde and van der Bruggen (1997) in Curr. Opin. Immunol. 9:684-693, Sahin *et al.* (1997) in Curr. Opin. Immunol. 9:709-716, and Shawler *et al.* (1997). These proteins have been shown to associate with certain tumors such as melanoma, lung cancer, prostate cancer, breast cancer, renal cancer and others.

A number of known antigens derived from pathogens can also be employed according to the present invention and is called "an infection-related protein". Pathogens contemplated by the present invention include a parasite, a yeast, a fungi, a bacterium or a virus. Specific examples of antigens characteristic of a pathogen include the influenza virus nucleoprotein (residues 218-226, as set forth in F. et al. (1997) J. Virol. 71: 2715-2721) antigens from Sendai virus and lymphocytic choriomeningitis virus (see, An et al. (1997) J. Virol. 71: 2292-2302), the B1 protein of hepatitis C virus (Bruna-Romero et al. (1997) Hepatology 25: 470-477), the virus envelope glycoprotein gp 160 of HIV (Achour et al. (1996) J. Virol. 70: 6741-6750), amino acids 252-260 or the circumsporozite protein of *Plasmodium berghei* (Allsopp et al. (1996) Eur. J. Immunol. 26: 1951-1958), the influenza A virus nucleoprotein (residues 366-374, Nomura et al. (1996) J. Immunol. Methods 193: 4149), the listeriolysin O protein of *Listeria monocytogenes* (residues 91-99, An et al. (1996) Infect. Immun. 64: 1685-1693), the E6 protein (residues 131-140, Gao et al. (1995) J. Immunol. 155: 5519-5526) and E7 protein (residues 21-28 and 48-55, Bauer et al. (1995) Scand. J. Immunol. 42: 317-323) of human papillomavirus type 16, the M2 protein of respiratory syncytial virus (residues 82-90 and 81-95, Hsu et al.

(1995) Immunology 85: 347-350), the herpes simplex virus type 1 ribonucleotide reductase (see, Salvucci et al. (1995) J. Gen. Virol. 69: 1122-1131) and the rotavirus VP7 protein (see, Franco et al. (1993) J. Gen. Virol. 74: 2579-2586), *P. falciparum* antigens (causing malaria) and hepatitis B surface antigen (Gilbert et al. (1997) Nature Biotech. 15: 1280-1283).

Accordingly, sequences coding for the above-described proteins may be cloned into the present expression vector for delivery.

A number of coding sequences for small antigenic peptides can also be employed in the present invention. One skilled in the art can readily determine the length of the fragments required to produce immunogenic peptides. Alternatively, the skilled artisan can also use coding sequences for peptides that are known to elicit specific T cell responses, such as tumor-associated antigenic peptides (TRAs) as disclosed by US5,965,381.

Antigenic peptides of a pathogen origin can also be used, such as those disclosed by Gilbert *et al.* (1997).

In the present invention said heterologous gene may code for a protein comprising the active subunit of a toxin whereby said toxin is chosen from the group comprising the diphteria toxin (dtxA), the cholera toxin (A1) and the antraxtoxin (LF and EF).

The diphteria toxin consists of two subunits, subunit A and subunit B. The fragment A of the diphteria toxin inhibits protein synthesis by ADP-ribosylation of elongation factor 2. It is normally delivered into eukaryotic cells by fragment B. Without fragment B, it is non-toxic, unless it is delivered into cells by another means. The inventors expressed this dtxA gene and proved that *Yersinia* can transfer this heterologous protein into several eukaryotic cell types (Boyd *et al.* 2000).

The cholera toxin is an oligomeric protein with a mass of 85,620 Daltons, with a total of 755 amino acid residues. The toxin is made of an A-subunit and 5 identical B subunits. This toxin binds to ganglioside G_{M1} receptors in the small intestine. It is then cleaved, and the enzymatic subunit enters the cell leading to a rise of intracellular cAMP. This results in the hyperactivity of Na^+ pumps, leading to diarrhea. When the A-subunit of the cholera toxin is inserted into the cell is cleaved along a disulfide bridge between A1 and A2, which keeps the protein inactive. (Merritt 1994). The A1 subunit of cholera toxin is the enzymatically active portion of the protein molecule, and it acts as an ADP-ribosyltransferase. Ribosylation of Gs stabilizes the GTP bound form of the protein, lower its GTPase activity creating a near constitutively on signal for the generation of

adenylyl cyclase, and therefore elevating cAMP levels. In the present invention, the inventors suggest to use the A1 subunit of the CTX to kill target cells using the *Yersinia* type III system.

Virulent strains of *Bacillus anthracis* produce three distinct antigenic components related to a complex exotoxin called the anthrax toxin. Each component of the toxin is a thermolabile protein with a MW of approximately 80kDa. Factor I is the edema factor (EF) which is necessary for the edema producing activity of the toxin. EF is known to be an inherent adenylate cyclase, similar to the *Bordetella pertussis* adenylate cyclase toxin. Factor II is the protective antigen (PA) or binding (B) domain of the anthrax toxin carrying the two active (A) domains, EF (above) and LF (below). Factor III is known as the lethal factor (LF) because it is essential for the lethal effects of the anthrax toxin. Apart from their antigenicity, each of the three factors separate exhibits no significant biological activity in an animal. However, combinations of two or three of the toxin components yield the following results in experimental animals: PA+LF combine to produce lethal activity, EF+PA produce edema and PA+LF+EF produces edema and necrosis and is lethal. EF+PA has been shown to elevate cyclic AMP to extraordinary levels in susceptible cells. Changes in intracellular cAMP are known to affect changes in membrane permeability and may account for edema. The present inventors suggest the use of LF and/or EF in combination with a *Yersinia* vector strain according to the present invention. PA is a carrying molecule and is not needed when using an alternative delivery system such as described *Yersinia* system.

Alternatively, said heterologous protein of the present invention is a drug. According to a preferred embodiment of the present invention, said drug is an anti-inflammatory compound. Preferentially, according to the present invention said anti-inflammatory compound is selected from the group comprising yopP or any intracellular compound inhibiting the NFkB-CREB pathway.

YopJ of *Yersinia pseudotuberculosis* inhibits multiple signal transduction pathways that converge to induce phosphorylation of the transcrition factor CREB (cAMP response element-binding protein). This transcription factor is intimately linked to cell fate in many different tissues. Meijer *et al.* (2000) propose YopJ as an antagonist circumventing innate and adaptive immune response at multiple levels. It was suggested to provide new avenues for therapeutic intervention in diseases where there is a need to block chronic inflammatory diseases. YopP is the YopJ homologue in *Y. enterocolotica*. The present inventors have shown that when one of the proteins is injected into animal cells

strongly induces anti-inflammatory reaction; this protein prevents the release of cytokine tumor necrosis factor alpha (TNFα), a cytokine that is central in the onset of the inflammatory response (BOLAND, A. and CORNELIS, G.R. Role of YopP in suppression of TNFα release by macrophages during *Yersinia* infection. Infection and Immunity, 1998, 66:1878-1884).

The present inventors suggest according to the present invention that live *Y.enterocolitica*, disarmed from all the Yop effectors but overproducing YopP could be used to treat inflammatory diseases.

The inhibition of NFκB activation is accompanied by a lack of activation of the mitogen-activated protein (MAP) kinases (MAPKs), c-Jun-N-terminal kinase (JNK), p38, and extracellular signal regulated kinase (ERK) 1 and 2 that is observed upon infection of macrophages by *Yersinia* producing YopP. Lack of activation of these MAPKs results from the inhibition of the upstream MAPK kinases (MAPKKs) by binding of YopP (Cornelis 2000). According to the present invention, said intracellular compound inhibiting the NFκB-CREB pathway may be chosen from the group comprising IκB and MAPKK-, MAPK-, JNK-, p38-, or ERK- inhibitors.

One preferred embodiment of the present invention is directed to a *Yersinia* of the above-described mutant *Yersinia* strain transformed with an expression vector for delivery of a heterologous protein as described above into a target cell.

Sequences coding for a full-length naturally occurring protein, a part of a naturally occurring protein, combinations of parts of a naturally occurring protein, or combinations of different naturally occurring proteins or parts from different proteins, may all be employed in the present invention. For example, a sequence coding for multiple functions may be used.

Those skilled in the art are familiar with the techniques to make DNA fragments coding for a part of a protein, or link a DNA sequence encoding a part of one protein in frame to a DNA sequence encoding a part of another protein and the like.

The vectors of the present invention may include other sequence elements such as a 3' termination sequence (including a stop codon and a poly A sequence), or a gene conferring a drug resistance which allows the selection of *Yersinia* transformants having received a vector according to the present invention.

The expression vectors of the present invention may be transformed by a number of known methods into *Yersinia*. For the purpose of the present invention, the methods of transformation for introducing an expression vector include, but are not limited to,

electroporation, calcium phosphate mediated transformation, conjugation, or combinations thereof. For example, a vector can be transformed into a first bacteria strain by a standard electroporation procedure. Subsequently, such a vector can be transferred from the first bacteria strain into *Yersinia* by conjugation, a process also called "mobilization". *Yersinia* transformant (i.e., *Yersinia* having taken up the vector) may be selected, e.g., with antibiotics. These techniques are well known in the art. See, for example, Sory *et al.* (1994). Nevertheless, after the strain has been selected, the antibiotic resistance needs to be removed this to keep the *Yersinia* strict to the definition of a safe vector strain as defined by the present invention.

Accordingly, the present invention contemplates a method for delivering heterologous proteins as herein above described into eukaryotic cells.

By "target", is meant the extracellular adhesion of *Yersinia* to an eukaryotic cell. The present invention contemplates a wide range of eukaryotic cells that may be targeted by the instant recombinant *Yersinia.*

As described herein above, a first step in creating a non-pathogenic, non-adherent strain according to the present invention is to eliminate invasin and/or YadA which normally trigger interaction with target cells. This result in a strain that is not able to transfer any protein to any target cells anymore. The present invention further suggest that in a second step said *Y. enterocolitica* polymutant can be targeted specifically to target cells by labeling these cells with a specific targeting signal. Therefore, the present invention relates in particular to a safe non-virulent *Yersinia enterocolitica* mutant strain carrying mutation in at least one of the effector genes (YopH, yopO, yopP, yopE, yopM, yopT), in at least one additional mutation in the invasin genes (yadA, inv) and which is specifically modified with a targeting signal allowing a cell specific interaction of said *Yersinia* strain with a specific cell type. The present invention also gives some evidence that addition of the targeting signal 'as such' without additional deletion of the invasin genes improves already major the specific delivery of proteins into target cells.

Therefore present invention relates generally to a *Yersinia enterocolitica* mutant strain which is specifically modified with a targeting signal allowing a cell specific interaction of said *Yersinia* strain with a specific cell type.

According to the present invention said targeting signal, which is carried by a *Yersinia enterocolitica* mutant according to the present invention, may be a protein, a peptide, a lipid or a combination thereof. According to the present invention, said targeting signal is carried by a bacterial surface display system. There are a variety of bacterial surface

display systems for peptides. These can be the presented outside the cell via pili, autotransporters, lpp-ompA and LamB. Some of these surface display systems have proved useful for controlled bacterial adhesion (eg. type I pili and lpp-ompA) to inorganic and organic surfaces (such us ZnO and cellulose).

According to the present invention said displayed peptides may be scFv and small protein domains.

Some scFv and protein domains show high affinity to surface markers found in the target cell. Efficiency of recognition between vector cell and target cells is determined by the interaction efficiency of both compounds, the peptide exposed by the recombinant vector strain and the receptor thereof present on the target cell. The higher the efficiency, the faster the interaction will occur and the lower the percentage of non-specific interactions will be. The virulon system is dependent on this contact and/or regulated under specific promoters activated by contact- or intracellular bacterial growth as described for the virulon system of type III bacteria.

The inventors determined that antibodies recognizing the cell markers could be engineered onto the surface delivery system of in *Yersinia* resulting in a high affinity antigen-antibody reaction; proving that the adherence is strong enough to promote delivery. In this study the inventors analysed the transfer of YopE or Cya. YopE disrupts actin filaments and provokes a very clear cytopathic effect which is easy to monitor; transfer of the Cya reporter results in an accumulation of cyclic AMP which can be assayed by a routine, commercial ELISA test in the target cell allowing quantification of the translocation.

According to the present invention, said targeting signal carried by a *Yersinia enterocolitica* mutant according to the present invention strain may be a bacterial adhesin. In a preferred embodiment of the present invention said bacterial adhesin is selected from the group comprising Opa-proteins and Afa-proteins.

Neisseria meningitidis shows a specific predilection for binding to the leptomeninges and meningeal blood vessels in human brain and not to the cerebral cortex. The major ligand that mediates adherence is the pilus. The presence of Opa protein increases the association of Cap+ meningococci that expressed low-adhesive pili, but do not influence the association of high-adhesive pili (Hardy *et al.* 2000) *Mol Microbiol* 2000 May;36(4):817-29). A number of neisserial adhesins (i.e. pilli, Opa, Opc and P36) and additional putative virulence determinants which affect bacterial adherence and invasion into host cells (i.e. LOS, capsule, PorB) have been identified. Clearly, neisserial surface

variation serves as an adaptive mechanism which can modulate tissue tropism, immune evasion and survival in the changing host environment. Important progress has been made in recent years with respect to the host cellular receptors and subsequent signal transduction processes which are involved in neisserial adherence, invasion and transcytosis. This has led to the identification of (i) CD46 as a receptor for pilus which allows adherence to epithelial and endothelial cells, (ii) HSPGs, in cooperation with vitronectin and fibronectin, as receptors for a particular subset of Opa proteins and Opc, which may both mediate invasion into most epithelial and endothelial cells, and (iii) CD66 as the receptors for most Opa variants, potentially being involved in cellular interactions including adherence, invasion and transcytosis with epithelial, endothelial and phagocytic cells. (reviewed by Dehio et al. *Subcell Biochem* 2000;33:61-96. Therefore, the present inventors suggest to use this Opa protein as targeting protein on a mutant recombinant vector strain to target epithelial, endothelial and phagocytic cells.

The afimbrial adhesive sheath of *Escherichia coli*, encoded by the afa-3 gene cluster, is composed of two proteins with different roles in bacterium-HeLa cell interactions. AfaE is required for adhesion and AfaD for internalization. AfaD is the prototype of a family of invasins encoded by adhesion-associated operons in pathogenic *E. coli*. (Garcia *et al FEBS Lett* 2000 Aug 18;479). The present inventors suggest to use this AfaE protein as targeting protein on a mutant recombinant vector strain to target human intestinal cells cells, since this adhesins recognizes the brush-border-associated decay-accelerating factor (DAF ; CD55).

Alternatively, the *Yersinia enterocolitica* mutant strain according to the present invention may carry a specific targeting signal chosen from antibodies able to recognize a specific cell marker.

According to a preferred embodiment of the invention, said cell specific marker recognized by the targeted *Yersinia enterocolitica* mutant strain is selected from the group comprising members of tumor–antigen, parasite-specific antigen. Examples of said proteins are given in previous paragraphs. Cell surface markers of human colon cancer, at early and late stages, have been identified. For instance, colonic cancer cells could be targeted with antibodies directed against specific surface antigens such as Ep-CAM, carcinoembryogenic antigen (CEA), STEAP or A33.

Further the present invention relates to a *Yersinia enterocolitica* mutant strain as defined above (see any of claims 14 to 20) and comprising mutations as defined above (see any of claims 1 to 4).

The present invention further contemplates a method for delivering a heterologous protein into a target cell comprising contacting said target cell with a *Yersinia* mutant strain according to the present invention.

Preferably, the method according to the present invention allows the delivery of a heterologous protein into a target cell wherein said target cell is an eukaryotic cell of plant, human, animal or parasitic origin. Further in accordance with the present invention, the delivery of a protein can be achieved by contacting an eukaryotic cell with a recombinant *Yersinia* under appropriate conditions. Various references and techniques are conventionally available for those skilled in the art regarding the conditions for inducing the expression and translocation of virulon genes, including the desired temperature, Ca++ concentration, manners in which *Yersinia* and target cells are mixed, and the like. See, for example, Cornelis, Cross talk between *Yersinia* and eukaryotic cells, Society for General Microbiology Symposium, 55; MoCRAE, SAUNDERS, SMYTH, STOW (eds), Molecular aspects of host-pathogen interactions, Cambridge University Press, 1997. The conditions may vary depending on the type of eukaryotic cells to be targeted, e.g., the conditions for targeting human epithelial carcinoma Hela cells (Sory et al. (1994)); the conditions for targeting mouse thymoma or melanoma cells (Starnbach et al. (1994) J. Immunol. 153: 1603);.the conditions for targeting mouse macrophages (Boland et al. (1996)). Such variations can be addressed by those skilled in the art using conventional techniques.

Those skilled in the art can also use a number of assays to determine whether the delivery of a fusion protein is successful. For example, the fusion protein may be labeled with an isotope or an immunofluoresceine, or detected by a immunofluoresceine conjugated antibody, as disclosed by Rosqvist et al. (1994) EMBO J. 13: 964. The determination can also be based on the enzymatic activity of the protein being delivered, e.g., the assay described by Sory et al. (1994). The determination can also be based on the antigenicity of the protein being delivered. For example, the delivery of a MAGE-1 protein into EBV-transformed human B cells can be detected by the recognition of such targeted B cells by CTL cells specific for MAGE-1 epitopes. Such CTL recognition, in turn, may be detected by a number of assays including assaying the secretion of IFN-.gamma. from the activated CTLs or Cr@51 release from lysed target cells. Methods such as Western-blot analysis using antibodies specific against the protein being delivered, PCR in situ hybridization, or ELISPOT (Mabtech AB, Sweden) may also be

employed for such determination. See, e.g., W. Herr et al. (1997) J. Immunol. Methods 203: 141-152 and W. Herr et al. (1996) J. Immunol. Methods 191: 131-142.

More specifically, said eukaryotic target cell may be selected from the group consisting of an antigen presenting cell, a cancer cell, an infected cell or an inflamed cell.

In particular, the present invention contemplates antigen-presenting cells. "Antigen presenting cells" as referred herein express at least one class I or class II MHC determinant and may include those cells which are known as professional antigen-presenting cells such as macrophages, dendritic cells and B cells. Other professional antigen-presenting cells include monocytes, marginal zone Kupffer cells, microglia, Langerhans' cells, interdigitating dendritic cells, follicular dendritic cells, and T cells. Facultative antigen-presenting cells can also be used according to the present invention. Examples of facultative antigen-presenting cells include astrocytes, follicular cells, endothelium and fibroblasts. As used herein, "antigen-presenting cells" encompass both professional and facultative types of antigen-presenting cells.

In a further aspect of the present invention, recombinant *Yersinia* capable of delivering proteins to antigen-presenting cells are employed for inducing an immune response. Accordingly, the present invention contemplates immunogenic compositions and methods for inducing specific immune responses using the recombinant *Yersinia* according to the present invention as described herein above.

The immune responses contemplated by the present invention include cellular immune responses (mediated primarily by T cells) and humoral immune responses (mediated primarily by antibodies). Janeway and Travers teach in general these immune response. (Janeway and Travers (1996) Immunology, The Immune System in Health and Disease 2nd ed. Garland Publishing, Inc.: New York, N.Y., and London, England.) (See also, review by O. Tureci et al. (1997) Molecular Medicine Today 3(8): 342-349.

The antigen presenting cells can be isolated from tissue or blood (containing peripheral blood mononuclear cells) samples obtained from a mammal such as a human or rodent. Cell lines established from such samples may also be used. Procedures for establishing cell lines are well known in the art. Certain cell lines may be obtained directly from the American Type Culture Collection, 10801 University Boulevard, Manassas, Va. 20110-2209. Both normal and malignant cells may be employed.

In accordance with a preferred embodiment of the present invention, the MHC determinants expressed by the antigen presenting cell are compatible with those expressed by the mammal involved, and at least one of these MHC determinants is

capable of presenting one or more antigenic epitopes derived from the protein being delivered.

One skilled in the art is also familiar with the methods for determining whether the MHC molecules expressed by the antigen presenting cell are compatible with those of the mammal subject involved, such as well known HLA-typing procedures. See general teachings by Coligan et al. (1994) Current Protocols in Immunology John Wiley & Sons Inc: New York, N.Y.

Those skilled in the art are able, through the extensive teachings in the art, to determine the MHC molecule for presentation of a particular antigen. For example, U.S. Pat. No. 5,405,940 teaches the determination of HLA-A1 as the presenting molecule for a peptide of MAGE-1, EADPTGHSY; U.S. Pat. No. 5,558,995 teaches the determination of HLA-Cw1601 for presenting another peptide of MAGE-1, SAYGEPRKL; U.S. Pat. No. 5,530,096 teaches the determination of HLA-A2 as the presenting molecule for a peptide of Tyrosinase, MLLAVLYCL. In the event the eukaryotic cells being targeted do not express a desired HLA or MHC molecule, the gene encoding such molecule may be introduced into the eukaryotic cells by well known transformation or transfection procedures.

With "infected cell" is meant a cell which carries bacteria, parasites, yeast, fungus or a virus; "inflamed cell" indicates that within the cell a inflammatory pathway is activated such as found in autoimmune diseases.

The present invention also relates to a composition for use as a medicament or a cell based product intended for clinical use comprising a *Yersinia enterocolitica* mutant strain as described above. The pathological conditions contemplated by the present invention which can be targeted by the composition according to the present invention include tumors and infections by pathogens such as bacteria, parasites, yeast, fungus or virus. By "treating", is meant alleviating or inhibiting a pathological condition, e.g., inhibiting tumor growth or metastasis, reducing the size of tumor, or diminishing symptoms of a pathogen infection. The recombinant *Yersinia* of the present invention can also be employed *in vivo*, i.e., introducing recombinant *Yersinia* into a mammal, such as a human or rodent subject.

In particular, the present invention also contemplates the use of a composition for the preparation of a medicament for treating cancer, infections and inflammatory diseases. Said inflammatory disease may be an autoimmune diseases. The present invention also

provides a method for treatment of cancer, infections and autoimmune diseases comprising the use of said composition.

According to the present invention, said method can be applied for treating cancer comprising administering to a subject in need of treatment a therapeutically effective amount of a composition according to the invention, wherein the antigen is a tumor specific antigen. In this approach the *Yersinia* vector strain provides antigens to antigen presenting cells allowing the triggering of a T-cell immune response. This allows the activation of a complex machinery resulting in the elimination of the tumor carrying this specific tumor antigen.

A safe recombinant *Yersinia* may be employed in an immunogenic composition to induce an immune response for treating various pathological conditions in mammals. The pathological conditions contemplated by the present invention include tumors and pathogen infections, as disclosed herein.

Alternatively, said method can also be applied for treating cancer comprising administering to a subject in need of treatment a therapeutically effective amount of a composition according to the present invention, wherein a heterologous toxin is made and delivered to cancer cells. In this approach cancer cells are directly targeted, this in contrast to previous approach whereby the immunological response is responsible for the elimination of the cancer cells. According to the present invention a "killer" *Yersinia* enterocolitica only adheres to the cancer cells and not to any other cell. Said *Y. enterocolitica* polymutant, devoid of any effector but producing a lethal toxin, such as the diphteria toxin, can be used to approach and kill specific cancer cells. In addition, said method can be applied for treating infections comprising administering to a person in need of treatment a therapeutically effective amount of a composition according to the present invention, wherein the antigen is an infectious specific antigen. T-cells can be triggered by antigen presenting cells carrying the specific antigen provided by the vector strain of the present invention. In this way infected cells can be recognized by the immune response and eliminated. Alternatively, said method can be used for treating infections comprising administering to a person in need of treatment a therapeutically effective amount of a composition according to the present invention, wherein a heterologous toxin is made and delivered to said infected cells. This is an alternative approach compared to previous method. In this method the infected cells are targeted directly and does not need the activation of the immune system. When the vector strain expresses (a) toxin(s), said method allows the killing of a target cell comprising

contacting the target cell with said *Yersinia enterocolitica* mutant strain or a composition. Here the target cell may be a cancer cell, an infected cell, an activated cell of the immunological system (autoimmune diseases).

The method according to the present invention also provides the possibility to treat autoimmune disease comprising administering to a subject in need of treatment a therapeutically effective amount of a composition according to the present invention, wherein the antigen is a self protein linked to a MHC-factor. In this way, immune cells which are presenting self-proteins are eliminated, resulting in the decrease of the autoimmune response. Self-proteins as such are not recognized. Alternatively, the method according to the present invention can be used to treat inflammatory diseases comprising administering to a person in need of treatment a therapeutically effective amount of a composition according to the present invention, wherein an anti-inflammatory compound or toxin is expressed in the vector strain and delivered to said inflamed cells. Here also, the targeting is direct and allows the direct elimination of inflamed cells. The anti-inflammatory compound used is yopP or an intracellular compound inhibiting the NFkB-CREB pathway. In this way, the pathway, which normally results in the activation of the immune cells, is inhibited.

Until now, the composition according to the present invention is used to have a direct effect on the diseased state. Alternatively, this composition can also be used as a vaccine adjuvant. In this approach, the main compound that activates the immune system is different from the composition of the present invention; this latter is added to have a cooperative effect on the first. This vaccine adjuvant comprises a composition according to the present invention. The present invention also discloses the method for immunizing against a disease in humans or animals comprising administering a vaccine comprising an adjuvant of the present invention.

A "vaccine" is an immunogenic composition capable of eliciting protection against infections, whether partial or complete. A vaccine may also be useful for treatment of an individual, in which case it is called a therapeutic vaccine. Said vaccine compositions may include prophylactic as well as therapeutic vaccine compositions. The term "therapeutic" refers to a composition capable of treating infections. "Prophylactic" refers to a composition for treating patients which are susceptible to infections in order to prevent coming infection.

The present invention further contemplates the administration of the composition of the present invention in the subject. This may be carried out orally or parenterally such

as topical (including ophthalmic), intraperitoneal, subcutaneous, intradermal, intrapeural, intrathecal, intramuscular, intralymphoidal or intratumoral administration.

For *in vivo* use of recombinant *Yersinia*, the safety can be tested in animals beforehand. In this case, the recombinant *Yersinia* may be administered to the animal orally or directly into the stomach. The animals may be sacrificed a few days (1-3 days) after the administration of the recombinant *Yersinia*. The intestines are washed and the Peyer patches or the faeces can be examined for viable *Yersinia*. See, e.g., Sory et al. (1992) Infect. Immun. 60: 3830-3836. The recombinant *Yersinia* may also be administered to the animal by intraperitoneal injection. Organs of sacrificed animals such as spleen and liver can be examined for the presence of intracellular *Yersinia*, an indication of insufficient safety. Intracellular *Yersinia* may be detected by e.g., cultivating cell extracts on solid medium. See teachings by Sory et al. (1988) Microb. Pathogen 4: 431-442.

Formulations for such administrations may comprise an effective amount of the composition according to the present invention in physiological saline or any buffer/media deprived from any possible allergen. When applied orally, the vector strain will remain in the digestive tract. This application therefore is focused on the cure of diseases located within the digestive tract. Preferentially, said part of the digestive tract is the colon. One of the frequently occurring autoimmune diseases of the digestive tract in humans is the Crohne disease.

The present invention also relates to the pharmacological composition comprising the compound according to the invention and optionally a pharmaceutical acceptable carrier, diluent or excipient. The immunogenic compositions can include, in addition to a recombinant Yersinia, other substances such as cytokines, adjuvants and pharmaceutically acceptable carriers. Cytokines can also be included in such immunogenic compositions using additional recombinant *Yersinia* of the present invention capable of delivering a cytokine, for example.

These compositions may, for example, be administered parentally or intravenously. The compositions according to the invention for parenteral administration can be, in particular, sterile solutions, aqueous or non-aqueous, suspensions or emulsions, As a pharmaceutically acceptable solution or vehicle propylene glycol, polyethylene glycol, injectable organic esters, for example ethyl oleate, or cyclodextrins may be employed. These compositions can also comprise wetting, emulsifying and/or dispersing agents.

They may also be prepared in the form of sterile solid compositions which may be dissolved at the time of use in sterile water or any other sterile injectable medium.

The present invention can also comprise adjuvants which are well known to a person skilled in the art (vitamin C, antioxidant agents etc.) capable of being used in synergy with the compounds according to the invention in order to improve and prolong the treatments of the diseases.

Compositions can be provided together with physiologically tolerable liquid, gel or solid carriers, diluents, adjuvants and excepients. Formulations may contain such normally employed additives as binders, fillers, carriers, preservatives, stabilizing agents, emulsifiers, buffers and excipients as for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, cellulose, magnesium carbonate and the like. These compositions contain 1%-95% of active ingredient, preferably 2%-70%.

The compositions are preferentially prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or in suspension in, liquid prior to injection may also be prepared.

The composition of the present invention are often mixed with diluents or excipients which are compatible and physiologically tolerable. Suitable diluents and exipients are for example, water, saline, dextrose, glycerol, or the like, and combinations thereof. In addition, if desired the compositions may contain minor amounts of auxiliary substances such as wetting or emulsifying, stabilizing or pH buffering agents.

The present invention also relates to a method to produce a safe non-virulent *Yersinia enterocolitica* mutant strain for delivering heterologous proteins in target cells comprising the transformation of polymutant cells carrying mutations in at least one of the effector genes (yopH, yopO, yopP, yopE, yopM, yopT) and at least one additional mutation in the invasin genes (yadA and/or inv) with an expression vector as defined by the present invention.

As described herein above, recombinant vector strains can be made by introducing expression vector in the corresponding vector strain. This final vector strain can be applied to deliver heterologous proteins/ DNA in a large variety of cells. According to the present invention, the production of said strain can occur *in vitro* or *in vivo*. Most probably, the recombinant strain will be made *in vitro* as described above, but it is not excluded that specific phages carrying the desired expression vector can be injected

together or separate from the target strain. This specific phage can infect the vector strain within the body of the subject.

The present invention also suggests the use of a *Yersinia enterocolitica* mutant strain according to the invention for the preparation of *in vitro* screening assays. Said *in vitro* screening method using a *Yersinia enterocolitica* mutant can be used in different respects.

Said method can be applied for detecting T cell mediated activity of a target antigenic peptide, comprising at least the following steps:

(a) providing a *Yersinia enterocolitica* mutant strain according to the invention carrying an antigenic peptide,

(b) contacting an antigen presenting cell with said *Yersinia* mutant strain, thereby providing an activated T cell,

(c) contacting a target cell with said activated T cell, and,

(d) monitoring the effect of said activated T cell on said target cell, thereby detecting anti-target activity.

In the design and conduct of above described applications, important considerations include methods for introducing the antigen into MHC class I and II processing pathways, methods for selecting the correct vector strain, route of administration and antigen selection. Because the cell therapy as presented in the present invention needs a specific recognition of the target cell, it is important that indeed the choice of antigen is well considered. Therefore the present invention suggests that the antigen is a tumor specific antigen, an infectious specific antigen or a self-protein-MHC-complex when applied in the treatment of cancer, infections (viral, bacterial, parasitical) or autoimmune diseases. In addition, it is important that the compositions are administered to a person in need of treatment in a therapeutically effective amount. Example of antigens that might be considered as tumor antigens are described by Fong and Engleman 2000[36].

Alternatively said method can be applied for detecting peptides or proteins interfering with the NFkB/CREB pathway, comprising at least the following steps: (a) providing a *Yersinia enterocolitica* mutant strain according to the invention carrying an peptide/protein peptide as agonist or antagonist candidate for the NFkB pathway, (b) contacting an target cell which has been activated using LPS or an alternative thereof with the recombinant vector strain. It is known that LPS activates the NFkB pathway, and, (c) monitoring the effect of said vector strain on said target cell, thereby detecting anti-inflammatory activity. According to this embodiment, the present invention provides

a method of identifying compounds (peptides or proteins) which selectively inhibit, induce or interfere with the expression/production of the polypeptides necessary in the NFkB pathway. Compounds may carry agonistic or antagonistic properties.

A cell-mediated immune response allows the natural elimination of specific cells. The specificity depends on the specificity of the antigen that is used to activate the antigen presenting cells. In this approach according to the present invention the antigen is delivered to the antigen presenting cell using a vector strain. Said strain expresses the antigen as heterologous protein. According to the present invention the method for inducing a cell-mediated immune response specific for a heterologous protein can occure *in vitro*, comprising the steps of: (a) selecting an antigen presenting cell expressing an MHC molecule capable of presenting at least one epitope of said heterologous protein; (b) forming a cell mixture by contacting said antigen presenting cell with a *Yersinia enterocolitica* mutant strain carrying mutations in at least one of the effector genes (yopH, yopO, yopP, yopE, yopM, yopT) and at least one additional mutation in the invasin genes (yadA and/or inv) and expressing an antigen, and, (c) contacting a sample containing peripheral blood lymphocytes taken from a subject, with the cell mixture formed in step (b) thereby inducing *in vitro*, a cell-mediated response specific for said heterologous protein. Recombinant Yersinia can also be employed in an *ex vivo* regime for inducing CTLs specific for a protein. The procedure to develop such specific CTLs in vitro is known in the art, e.g., as disclosed by the U.S. Pat. No. 5,342,774. Briefly, a blood sample containing T cell precursors is taken from a mammal. PBLs are purified from such blood sample and are incubated with stimulator cells expressing an antigenic epitope in the context of an MHC molecule. CTLs specific for such epitope produced can be detected by assays such as an assay for Cr@51 release or secretion of IFN-gamma.

According to the present invention, a mixture of a recombinant *Yersinia* and an antigen presenting cell can be used as the "stimulator cell" in such an *in vitro* procedure for producing CTLs specific for the protein being delivered. The MHC determinants expressed by the antigen presenting cell used are compatible with those expressed by the mammal from which PBLs are isolated, and at least one of these MHC molecules is capable of presenting, to T cells, one or more epitopes derived from the protein being delivered. CTL cells generated as such can be administered, in a therapy regimen of adoptive transfer, to a mammal a pathological condition characterized by an abnormal expression of the protein used in the delivery system. See teachings by Greenberg

(1986) J. Immunol. 136 (5): 1917; Riddel et al. (1992) Science 257: 238; Lynch et al. (1991) Eur. J. Immunol. 21: 1403; and Kast et al. (1989) Cell 59: 603 for adoptive transfer. CTLs, by lysing the cells abnormally expressing such antigens, can alleviate or treat the pathological condition at issue such as a tumor, an infection with a parasite or a virus.

Alternatively, the method for inducing a cell-mediated immune response specific for a heterologous protein can occur *in vivo*, comprising the steps of: (a) selecting a *Yersinia* mutant strain carrying mutations in at least one of the effector genes (yopH, yopO, yopP, yopE, yopM, yopT) and at least one additional mutation in the invasin genes (yadA and/or inv) and expressing an antigen; (b) forming a cell mixture by contacting said *Yersinia mutant* strain with an antigen presenting, and, (c) contacting peripheral blood lymphocytes with the cell mixture formed in step (b). In said method at least step (a) is performed *in vitro*; steps (c) and/or (d) may be performed *in vivo* by injecting the mutant stain (a) or the cell mixture (b) into a subject.

In all these approaches the induction of the immune system is needed. The antigen presenting cells absorb the recombinant *Yersinia* cells using their natural systems. As the inventors proved that the *Yersinia* does not have specific interaction with target cells, it might also be important to increase specificity of the *Yersinia* for the antigen presenting cell. Therefore the present invention also relates to a method where a cell-mediated immune response is elicted using a specific targeting system as described by the invention. In particular this invention relates to a method for inducing *in vitro*, a cell-mediated immune response specific for a heterologous protein, comprising the steps of: (a) selecting a recombinant *Yersinia enterocolitica* according to the invention expressing an MHC molecule presenting at least one epitope of a heterologous protein; (b) forming a cell mixture by contacting an antigen presenting cell with said *Yersinia enterocolitica* mutant strain, and, (c) contacting a sample containing peripheral blood lymphocytes taken from a subject, with the cell mixture formed in step (b) thereby inducing *in vitro*, a cell-mediated response specific for said heterologous protein.

The present invention also relates to a method for inducing *in vivo*, a cell-mediated immune response specific for a heterologous protein, comprising the steps of: (a) selecting a recombinant *Yersinia enterocolitica* according to the present invention expressing an MHC molecule presenting at least one epitope of a heterologous protein; (b) forming a cell mixture by contacting an antigen presenting cell with said *Yersinia enterocolitica* mutant strain, and, (c) contacting a sample containing peripheral blood

lymphocytes taken from a subject, with the cell mixture formed in step (b) thereby inducing *in vitro*, a cell-mediated response specific for said heterologous protein; whereby at least step (a) is performed *in vitro*; steps (b) and/or (c) may be performed *in vivo* by injecting the mutant stain (a) or the cell mixture (b) into a subject.

The present invention also further contemplates a method for monitoring a cellular immune response in a subject before, during and after a vaccination regimen, comprising the steps of: (a) obtaining from said subject an antigen presenting cell expressing an MHC molecule; (b) forming a cell mixture by contacting said antigen presenting cell with a *Yersinia* strain according to the present invention, wherein said second DNA sequence in the expression vector codes for at least one epitope of said antigen which is presented by said MHC molecule of said antigen presenting cell; thereby delivering said heterologous protein into said antigen presenting cell; and (c) contacting a sample containing peripheral blood lymphocytes taken from said subject, with the cell mixture formed in step (b), and assaying for the presence of a cell-mediated immune response specific for said antigen thereby monitoring a cell-mediated immune response in said subject before, during and after a vaccination regimen. According to this aspect of the present invention, the immune responses induced with the recombinant *Yersinia* can be utilized in a number of regimes for diagnostic or therapeutic use. For example, recombinant *Yersinia* can be employed in an *in vitro* procedure for monitoring the efficacy of a vaccination therapy in a mammal such as a human or rodent. In this regime, certain antigen presenting cells (e.g., dendritic cells) are taken from a subject being vaccinated with immunogenic compositions, e.g., a particular antigen. Such antigen presenting cells are then contacted with recombinant *Yersinia* capable of delivering the antigen which is used for vaccination. Subsequently, peripheral blood lymphocytes taken from the same subject (i.e., autologous PBLs) are added, preferably in combination with cytokines such as IL-2, to the mixture of antigen presenting cells and *Yersinia*. The efficacy of the vaccination can be assessed after priming and then after successive boosts by the presence of CTLs or antibodies that are specific for the relevant antigen. The presence of specific CTLs can be detected using standard assays such as an assay for Cr@51 release or for the secretion of IFN-gamma. The presence of specific antibodies can be detected by assays such as ELISA using the antigens which are immobilized on a culture plate, or a standard proliferation assay for T-helper cells.

In addition, the present invention also describes a method for detecting direct anti-target activity (anti-cancer or anti-inflammatory or anti-infectious activity) of a *Yersinia*

enterocolitica mutant strain as described by the invention, comprising at least the following steps: (a) providing a *Yersinia enterocolitica* mutant strain according to the invention, expressing a protein marker (b) contacting a target (cancer, infected or inflamed) cell with said *Yersinia* strain, (c) monitoring the effect of said *Yersinia* strain, on said target cell, thereby detecting anti-target activity.

The present invention also provides a kit for detecting T cell mediated activity of a target antigenic peptide or for detecting direct antitarget activity (anti-cancer or anti-inflammatory or anti-infectious activity), comprising at a *Yersinia enterocolitica* mutant strain according to the invention.

The present invention also relates to a secretion-type-III-carrying strain chosen from Pseudomonas (*P. aeroginosa*), Bordetella *(B. pertussis), Burholderia (B. cepacia), Chlamidia* strains or *Yersinia* (*Y. enterocolitica*, *Y. pseudotuberculosis*, or *Y. pestis*) carrying polymutations in equivalent genes as described for *Y. enterocolitica* carrying mutations in at least one of the effector genes (yopH, yopO, yopP, yopE, yopM, yopT) and at least one additional mutation in the invasin genes (yadA, inv).

The present invention also relates to an expression vector for delivering a heterologous protein into a eukaryotic cell using a secretion-type III-carrying strain carrying mutations in at least one of the equivalent effector genes (yopH, yopO, yopP, yopE, yopM, yopT) and at least one additional mutation in the equivalent invasin genes (yadA, inv) carrying elements as defined hertein above wherein said *Yersinia* sequence elements are exchanged by a sequence element or signal specific for the strain used or compatible therewith.

The present invention also relates to a secretion type III carrying bacterial strains according to present invention transformed with an expression vector according to present invention; its use for the delivery of heterologous proteins in a target cell as described herein for *Y enterocolitica*; a composition as described for *Y enterocolitica* according to present invention; its use for the preparation of a medicament usable in the treatment of various diseases as described for Y enterocolitica according to present invention, or its use in *in vitro* tests as described for *Y enterocolitica* according to present invention.

Brief description of the figure and Table

Figure 1: Effect of YopB, YopE, YopH, YopT on phagocytosis

Table 1: List of the strains and genotype.

Modes for carrying out the invention:

Example 1: Role of YopT and other Yops in prevention of phagocytosis

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table I. The strains were routinely grown in tryptic soy broth (TSB) and plated on tryptic soy agar (TSA) containing the required antibiotics. For *in vitro* induction of the yop genes, *Y. enterocolitica* was grown in brain heart infusion (BHI), supplemented with 20 mM sodium oxalate, 20 mM $MgCl_2$ and 0.4 % glucose (BHI-ox). Prior to cell infection, *Y. enterocolitica* was grown in non-supplemented BHI. Selective agents were used at the following concentrations: ampicillin 200 µg/ml, chloramphenicol 10 µg/ml, nalidixic acid 35 µg/ml, streptomycin 100 µg/ml, kanamycin 50 µg/ml, sucrose 5 % and 1 mM arsenite.

Molecular Biology techniques

Yops were precipitated from culture supernatants by ammonium sulfate (400 mg/ml) (Cornelis *et al.*, 1987), analysed by SDS-PAGE and where appropriate transfered to nitrocellulose membranes. Immunoblots were developed using a secondary antibody conjugated to horse-radish peroxidase and Supersignal (Pierce, Rockford, IL, USA) as chemiluminescent substrate. Mutator plasmids were introduced into *Y. enterocolitica* strains by conjugation from *E. coli* SM10λpir. After selection on appropriate media the mutations were confirmed by PCR analysis and by Yop induction and SDS-PAGE analysis of the secreted proteins or Western blot analysis of the bacterial proteins.

Infection procedures

J774 macrophages were aliquoted into 24-well plates the day before infection. On the day of infection the cells were washed with fresh culture medium. *Y. enterocolitica* strains were grown overnight in TSB at 22°C. They were inoculated in BHI at OD600=0.2, grown for 2 h at 22°C and pre-induced by incubating the cells during 30 min at 37°C. After harvesting and washing in prewarmed saline, the bacteria were added to the cells (moi depends on assay). The infections were carried out for 30 min at 37°. Then the cells were washed 3 times in culture medium after infection before proceeding.

Determination of bacterial uptake by immunofluorescence

To distinguish between intra- end extracellularly located bacteria, the double-immunofluorecence test described by Heesemann and Laufs (1984) was used. The presence of intracellular and extracellular bacteria was studied. When high percentages of extracellular bacteria were present indicated that phagocytosis was inhibited.

Briefly, 30 min after infection the cell cultures were washed and the cover slips were overlaid with a rabbit antiserum directed against yersinia LPS, diluted (1/500) in phosphate-buffered saline (PBS) and incubated for 30 min at 4°C. These antibodies only bind to extracellular bacteria. Monolayers were washed four times with PBS and fixed with ice-cold methanol for 90 s. After methanol removal, extracellular bacteria were labeled with Texas-red-conjugated goat anti-rabbit antiserum (1 g/ml) for 20 min at 37°C, followed by four washes in PBS. Both intra- and extracellular bacteria were then stained by incubation of cover slips for 1 h at 37°C with anti-LPS antiserum, washed in PBS and treated with fluorescein isothiocyanate-conjugated goat anti-rabbit antiserum. After four washes in PBS, cover slips were mounted in Mowiol mounting medium on a glass slide.

Extracellular bacteria were detected by exitation at 596 nm (Texas-red) and total cell-associated bacteria were detected at 490 nm (FITC). For each experiment, 50 cells per cover slips were randomly selected, and the numbers of extracellular and total cell-associated bacteria were determined. The experiments have been done three times in duplicate.

Result of Phacytosis assay with J774 macrophages cell line

To determine the potential roles of different *Yersinia enterocolitica* gene products in mediating phagocytosis, several *Y.enterocolitica* mutants were examined by immunofluorescence (see Figure 1).

The virulence plasmid-containing wild-type strain MRS 40 (pYV40) markedly inhibited the phagocytic capacity of the J774 cells. Of all the bacteria that became associated with the cells, more than 90% remained extracellularly bound. For the yopB-strain, unable to inject the different effector proteins in eukaryotic cells, only 18% were located outside. The ability of the yopH deletion mutant MRS40 (pSI4008) to inhibit phagocytosis was greatly reduced, less than 36% remained extracellular. Similar results were obtained for the yopE mutated strain MRS40 (pAB4052) where less than 45% remained extracellularly bound. The present inventors showed thar YopT, a 35-kDa protein, which is involved in cytoskeleton alteration and actin filament structure disruption (Iriarte and Cornelis, 1998) is also involved in antiphagocytic effect of *Yersinia*. Indeed,

50% of associated bacteria are phagocytosed in a yopT deletion mutant MRS40 (pIM409).

When the inventors studied the phagocytosis of double mutant of these three genes they observed each time that the phagocytosis percentage is higher than for the single mutant and lower than the triple mutant (data not shown).

Hence, YopH YopE and YopT are involved in phagocytosis resistance to macrophages but not sufficient to have alone a total antiphagocytic effect.

The foregoing example is illustrative of the invention and is not intended to limit the scope of the invention as set out in the claims. All of the references cited herein are incorporated by reference.

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Table 1

List of the strains and genotypes

Bacterial strains	genotype	references
MRS40(pAB404)	$yopE_{21}$, $yopH_{\Delta 1-352}$	Boland and Cornelis, 1998
MRS40(pIM424)	$yopE_{21}$, $yopT_{135}$	Iriarte and Cornelis, 1998
MRS40(pIM425)	$yopH_{\Delta 1-352}$, $yopT_{135}$	Boyd et al., 2000
MRS40(pIM426)	$yopE_{21}$, $yopH_{\Delta 1-352}$, $yopT_{135}$	Boyd et al., 2000
MRS40(pPW401)	$yopB_{\Delta 89-217}$	Boland et al., 1996
MRS40(pSI4008)	$yopH_{\Delta 1-352}$	Mills et al., 1997
MRS40(pYV40)	wild type	Sarker et al., 1998a
MRS40(pAB4052)	$yopE_{21}$	Boland and Cornelis, 1998
MRS40(pIM409)	$yopT_{135}$	Iriarte and Cornelis, 1998

CLAIMS

1. A safe non-virulent *Yersinia enterocolitica* mutant strain for delivering heterologous proteins in target cells carrying mutations in at least one of the effector genes yopH, yopO, yopP, yopE, yopM, yopT genes and at least one additional mutation in the invasin genes chosen from yadA and/or inv.

2. A safe non-virulent *Yersinia enterocolitica* mutant strain for delivering heterologous proteins in target cells according to claim 1 carrying mutations in all effector genes yopH, yopO, yopP, yopE, yopM, yopT genes and at least one additional mutation in the invasin genes chosen from yadA and/or inv.

3. A safe non-virulent *Yersinia enterocolitica* mutant strain for delivering heterologous proteins in target cells according to claims 1 or 2 further comprising an optional mutation present in at least one of the genes chosen from YopR, YopQ, YlpA, YomA, Yst, β-Lac or any gene linked to the iron acquisition system

4. A safe non-virulent *Yersinia enterocolitica* mutant strain for delivering heterologous proteins in target cells according to any of the claims 1 to 3 wherein said mutation is a mutation of the non-coding sequence of said gene and/or a mutation of the coding sequence of said gene.

5. An expression vector for delivering a heterologous protein into a target cell using a *Yersinia enterocolitica* mutant strain according to any of the claims 1 to 4, which comprises in the 5' to 3' direction:
   (a) a promoter of a *Yersinia* virulon gene,
   (b) a first DNA sequence encoding a delivery signal from a *Yersinia* effector protein, operably linked to said promoter; and,
   (c) a second DNA sequence coding for said heterologous protein, fused in frame to the 3' end of said first DNA sequence.

6. An expression vector according to claim 5 wherein said heterologous protein is a protein that is naturally occurring or a protein which is encoded by a gene but whereby the protein has never been demonstrated in nature.

7. An expression vector according to claim 6 wherein said heterologous protein, produced upon recombinant expression, is an antigen, a toxin or a drug.

8. An expression vector according to claim 7 wherein said heterologous protein is an antigen or at least one epitope of said protein is, whereby the antigen (epitope) is (is derived from) a tumor associated protein (TAA), an infection-related protein.

9. An expression vector according to claim 7 wherein said heterologous protein comprises the active subunit of a toxin, wherein said toxin is chosen from the group comprising the diphteria toxin (dtxA), the cholera toxin (A1) and the antraxtoxin (LF and EF).

10. An expression vector according to claim 7 wherein said drug (peptide/protein) is an anti-inflammatory compound.

11. An expression vector according to claim 10 wherein said anti-inflammatory compound is selected from the group consisting of yopP or an intracellular compound inhibiting the NFkB-CREB pathway.

12. An expression vector according to claim 11 wherein said intracellular compound inhibiting the NFkB-CREB pathway is chosen from the group comprising IκB and MAPKK-, MAPK-, JNK-, p38-, or ERK- inhibitors.

13. A *Yersinia enterocolitica* mutant strain according to any of the claims 1 to 4 transformed with an expression vector according to any of the claims 5 to 12.

14. A *Yersinia enterocolitica* mutant strain which is specifically modified with a targeting signal allowing a cell specific interaction of said *Yersinia* strain with a specific cell type.

15. A *Yersinia enterocolitica* mutant strain according to claim 14 wherein said targeting signal is a protein, a peptide, a lipid or a combination thereof.

16. A *Yersinia enterocolitica* mutant strain according to claim 14 wherein said targeting signal is carried by a bacterial surface display system.

17. A *Yersinia enterocolitica* mutant strain according to claim 15 wherein said targeting protein is a bacterial adhesin.

18. A *Yersinia enterocolitica* mutant strain according to claim 17 wherein said bacterial adhesin is selected from the group comprising OPA-proteins, AFA-proteins.

19. A *Yersinia enterocolitica* mutant strain according to claim 15 wherein said protein is an antibody recognizing a specific cell marker.

20. A *Yersinia enterocolitica* mutant strain according to claim 19 wherein said cell specific marker is selected from the group comprising a tumor–antigen or parasite-specific antigen.

21. A *Yersinia enterocolitica* mutant strain according to claim 14 to 20 comprising mutations as defined in any one of claims 1 to 4.

22. A method for delivering a heterologous protein into a target cell, comprising contacting said target cell with a *Yersinia* mutant strain according to any of the claims 1 to 4 or 13 to 21.

23. A method for delivering a heterologous protein into a target cell according to any of the claims 1 to 21 wherein said target cell is an eukaryotic cell of plant, human, animal or parasitic origin.

24. A method according to claim 23 wherein said eukaryotic target cell is selected from the group consisting of an antigen presenting cell, a cancer cell, an infected cell and an inflamed cell.

25. A method according to claim 24 wherein said antigen presenting cell is selected from the group consisting of a B cell, a macrophage, a dendritic cell, a monocyte, a follicular cell and a fibroblast.

26. A composition for use as a medicament or a cell based product intended for clinical use comprising a *Yersinia enterocolitica* mutant strain according to any of the claims 1 to 4 and 13 to 21.

27. A pharmaceutical composition comprising the compound according to claim 26 and optionally a pharmaceutical acceptable carrier, diluent or excipient.

28. Use of a composition according to claims 26 or 27 for the preparation of a medicament for treating cancer, infections and inflammatory diseases.

29. Use of a composition according to claims 26 or 27 wherein said inflammatory disease is an autoimmune disease.

30. Use of a composition according to claim 26 or 27 as a vaccine adjuvant.

31. Vaccine adjuvant comprising a composition according to claim 26 or 27.

32. A method for immunizing against a disease in humans or animals comprising administering a vaccine comprising an adjuvant of claim 31.

33. A method for treatment of cancer, infections or autoimmune diseases comprising the use of a composition according to claim 26 or 27.

34. A method for treating cancer comprising administering to a person in need of treatment a therapeutically effective amount of a composition of claim 26 or 27, wherein the antigen as defined in claim 20 is a tumor specific antigen.

35. A method for treating cancer comprising administering to a person in need of treatment a therapeutically effective amount of a composition of claim 26 or 27, wherein a heterologous toxin is made and delivered to cancer cells.

36. A method for treating infections comprising administering to a person in need of treatment a therapeutically effective amount of a composition of claim 26 or 27, wherein the antigen is an infectious specific antigen.

37. A method for treating infections comprising administering to a person in need of treatment a therapeutically effective amount of a composition of claim 26 or 27, wherein a heterologous toxin is made and delivered to said infected cells.

38. A method for treating autoimmune disease comprising administering to a person in need of treatment a therapeutically effective amount of a composition of claim 26 or 27, wherein the antigen is a selfprotein coupled to an MHC-factor.

39. A method for treating inflammatory diseases comprising administering to a person in need of treatment a therapeutically effective amount of a composition of claim 26 or 27, wherein an anti-inflammatory compound or toxin is made and delivered to said inflamed cells.

40. A method according to any of the claims 32 to 39 wherein said disease is located within the digestive tract.

41. A method according to claim 40 wherein said part of the digestive tract is the colon.

42. A method according to claim 41 wherein said disease is the Crohne disease.

43. A method of treatment according to any of claims 32 to 42, whereby the administration of the composition according to claim 26 or 27 in the patient is carried out orally and/or parenterally; whereby parenterally administration includes topical (including ophthalmic), intraperitoneal, subcutaneous, intradermal, intrapeural, intrathecal, intramuscular, intralymphoidal and/or intratumoral administration.

44. A method to produce a *Yersinia enterocolitica* mutant strain comprising the transformation of a polymutant strain according to any of the claims 1 to 4 with an expression vector as defined in claims 5 to 12.

45. A method for delivering a heterologous protein into a cell, whereby the production of a strain as described in claim 44 occurs *in vitro* or *in vivo*.

46. A method for killing a target cell comprising contacting said target cell with a *Yersinia enterocolitica* mutant strain or a composition according to claim 26 or 27.

47. Use of a *Yersinia enterocolitica* mutant strain according to any of claims 1 to 4 and 13 to 21 for the preparation of *in vitro* screening assays.

48. An *in vitro* screening method using a *Yersinia enterocolitica* mutant strain according to any of the claims 1 to 4 and 13 to 21.

49. A method for detecting T cell mediated activity of a target antigenic peptide, comprising at least the following steps:
(a) providing a *Yersinia enterocolitica* mutant strain according to any of the claims 13 to 21 carrying an antigenic peptide,
(b) contacting an antigen presenting cell with said *Yersinia* mutant strain,
(c) contacting a target cell with said activated T cell, and,
monitoring the effect of said activated T cell on said target cell, thereby detecting anti-target activity.

50. A method for detecting peptides or proteins interfering with the NFkB/CREB pathway, comprising at least the following steps:
(a) providing a *Yersinia enterocolitica* mutant strain according to any of the claims 13 to 21 carrying an peptide/protein peptide as agonist or antagonist candidate for the NFkB pathway,
(b) contacting an target cell which has been activated using LPS or an alternative thereof with the recombinant vector strain, and,
(c) monitoring the effect of said vector strain on said target cell, thereby detecting anti-inflammatory activity.

51. A method for inducing *in vitro*, a cell-mediated immune response specific for a heterologous protein, comprising the steps of:

(a) selecting an antigen presenting cell expressing an MHC molecule capable of presenting at least one epitope of said heterologous protein,

(b) forming a cell mixture by contacting said antigen presenting cell with a *Yersinia enterocolitica* mutant strain according to any of the claims 13 to 21 expressing an antigen, and,

(c) contacting a sample containing peripheral blood lymphocytes taken from a subject, with the cell mixture formed in step (b) thereby inducing *in vitro*, a cell-mediated response specific for said heterologous protein.

52. A method for inducing *in vivo*, a cell-mediated immune response specific for a heterologous protein, comprising the steps of:

(a) selecting a *Yersinia* mutant strain according to any of the claims 13 to 21 expressing an antigen,

(b) forming a cell mixture by contacting said *Yersinia mutant* strain with an antigen presenting, and,

(c) contacting peripheral blood lymphocytes with the cell mixture formed in step (b) whereby at least step (a) is performed *in vitro*; steps (c) and/or (d) may be performed *in vivo* by injecting the mutant stain (a) or the cell mixture (b) into a subject.

53. A method for inducing *in vitro*, a cell-mediated immune response specific for a heterologous protein, comprising the steps of:

(a) selecting a recombinant *Yersinia enterocolitica* according to any of the claims 13 to 21 expressing an MHC molecule presenting at least one epitope of a heterologous protein,

(b) forming a cell mixture by contacting an antigen presenting cell with said *Yersinia enterocolitica* mutant strain, and,

(c) contacting a sample containing peripheral blood lymphocytes taken from a subject, with the cell mixture formed in step (b) thereby inducing *in vitro*, a cell-mediated response specific for said heterologous protein.

54. A method for inducing *in vivo*, a cell-mediated immune response specific for a heterologous protein, comprising the steps of:

(a) selecting a recombinant *Yersinia enterocolitica* according to any of the claims 13 to 21 expressing an MHC molecule presenting at least one epitope of a heterologous protein,

(b) forming a cell mixture by contacting an antigen presenting cell with said *Yersinia enterocolitica* mutant strain, and,

(c) contacting a sample containing peripheral blood lymphocytes taken from a subject, with the cell mixture formed in step (b) thereby inducing *in vitro*, a cell-mediated response specific for said heterologous protein,

wherein at least step (a) is performed *in vitro*; and wherein steps (b) and/or (c) may be performed *in vivo* by injecting the mutant stain (a) or the cell mixture (b) into a subject.

55. A method for monitoring a cellular immune response in a subject before, during and after a vaccination regimen, comprising the steps of:

(a) obtaining from said subject an antigen presenting cell expressing an MHC molecule,

(b) forming a cell mixture by contacting said antigen presenting cell with a *Yersinia* strain according to claims 13 to 21, wherein said second DNA sequence in the expression vector codes for at least one epitope of said antigen which is presented by said MHC molecule of said antigen presenting cell; thereby delivering said heterologous protein into said antigen presenting cell, and,

(c) contacting a sample containing peripheral blood lymphocytes taken from said subject, with the cell mixture formed in step (b), and assaying for the presence of a cell-mediated immune response specific for said antigen thereby monitoring a cell-mediated immune response in said subject before, during and after a vaccination regimen.

56. A method for detecting direct anti-target activity (anti-cancer or anti-inflammatory or anti-infectious activity) of a *Yersinia enterocolitica* mutant strain as described in any of the claims 13 to 21, comprising at least the following steps:

(a) providing a *Yersinia enterocolitica* mutant strain according to any of the claims 23 to 28, expressing a protein marker,

(b) contacting a target cell with said *Yersinia* strain, and,

(c) monitoring the effect of said *Yersinia* strain, on said target cell, thereby detecting anti-target activity.

57. A kit for detecting T cell mediated activity of a target antigenic peptide or for detecting direct anti-target activity, comprising at a *Yersinia enterocolitica* mutant strain according to any of claims 1 to 4 and 13 to 21.

58. A secretion-type-III-carrying strain chosen from *Pseudomonas* (*P. aeroginosa*), Bordetella *(B. pertussis), Burholderia (B. cepacia), Chlamidia* strains or *Yersinia* (*Y. enterocolitica, Y. pseudotuberculosis*, or *Y. pestis*) carrying polymutations in equivalent genes as described for *Y. enterocolitica* according to any of the claims 1 to 4.

59. An expression vector for delivering a heterologous protein into a eukaryotic cell using a secretion-type III-carrying strain according to claim 58 carrying elements as defined in any of claims 5 to 22 wherein said *Yersinia* sequence elements are exchanged by a sequence element or signal specific for the strain used or compatible therewith.

60. A secretion type III carrying bacterial strain for the delivery of heterologous proteins into target cells according to claim 58 transformed with an expression vector according to claim 59.

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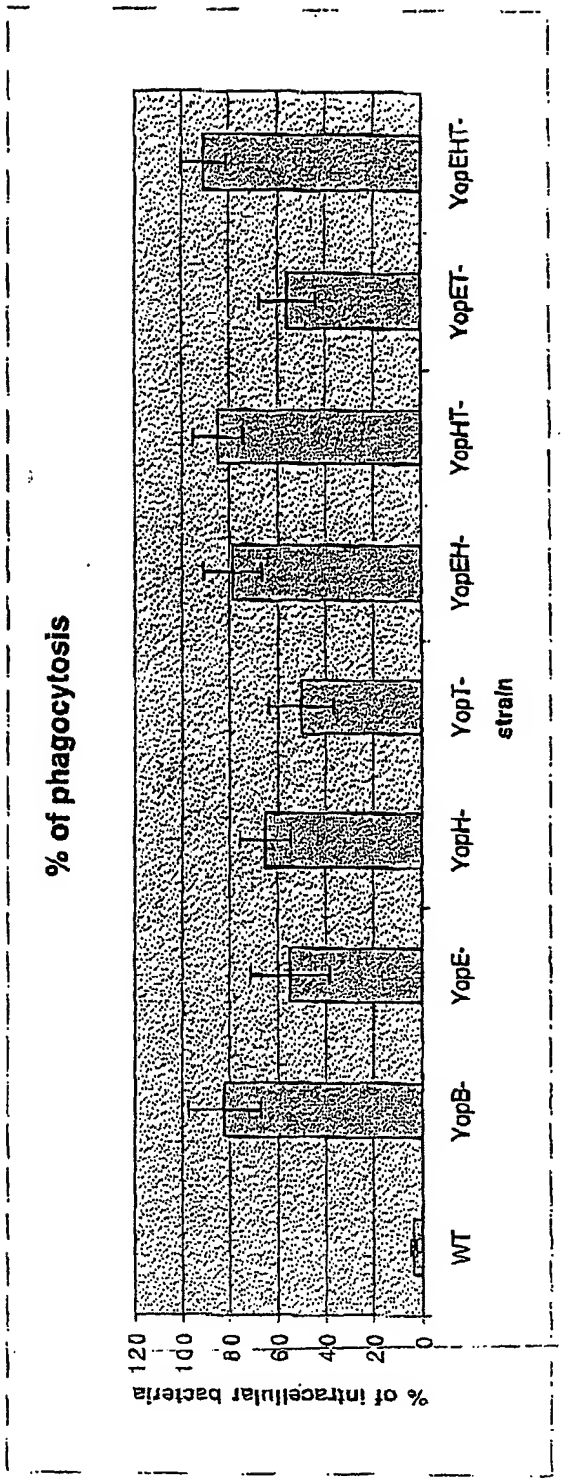
Figure 1

strain	%intra	SD
WT	3,66666667	1,10215885
YopB-	82,1428571	15,1653354
YopE-	54,7619048	16,6187345
YopH-	64,9325397	10,6729468
YopT-	50,1865079	13,5817477
YopEH-	78,4920635	12,1287645
YopHT-	84,5357143	10,4634912
YopET-	55,5952381	11,8137952
YopEHT-	90,5	9,54731956



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(54) Title: TYPE III BACTERIAL STRAINS FOR USE IN MEDICINE

(57) Abstract: The present invention relates to a safe non-virulent Yersinia enterocolitica mutant strain for delivering heterologous proteins in target cells carrying mutations in at least one of the effector genes yopH, yopO, yopP, yopE, yopM, yopT genes and at least one additional mutation in the invasin genes chosen from yadA and/or inv. The present invention also relates to a safe non-virulent Yersinia enterocolitica mutant strain for delivering heterologous proteins in target cells according to claim 1 carrying mutations in all effector genes yopH, yopO, yopP, yopE, yopM, yopT genes and at least one additional mutation in the invasin genes chosen from yadA and/or inv. The present invention also relates to an expression vector for delivering a heterologous protein into a target cell using a Yersinia enterocolitica mutant strain according to any of the claims 1 to 4, which comprises in the 5' to 3' direction :(a) a promoter of a Yersinia virulon gene, (b) a first DNA sequence encoding a delivery signal from a Yersinia effector protein, operably linked to said promoter; and, (c) a second DNA sequence coding for said heterologous protein, fused in frame to the 3' end of said first DNA sequence. The present invention further relates to methods and compositions comprising (the use of) the afore-mentioned mutant strains and expression vectors.

INTERNATIONAL SEARCH REPORT

In ional Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N1/36 C12N15/62 A61K35/74 C12R1/01 C12Q1/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K C12R C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BOYD A.P. ET AL.,: "Yersinia enterocolitica can deliver yop proteins into a wide range of cell types:develpoment of a delivery system for heterologous proteins" EUR. J. OF CELL BIOLOGY, vol. 79, - October 2000 (2000-10) pages 659-671, XP001013455	1,2,13, 22,57,58
Y	see whole doc. esp. p.663, 2.col.,2.par.fig.3, p.666 and discussion p. 669	3-60

Y	US 5 965 381 A (BOLAND ANNE M ET AL) 12 October 1999 (1999-10-12) cited in the application see whole doc. esp. claims	3-60

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search	Date of mailing of the international search report
2 October 2002	14/10/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mueller, F

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In ional Application No
PCT/EP 02/03372

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	MICHIELIS T ET AL: "Secretion of hybrid proteins by the Yersinia Yop export system" JOURNAL OF BACTERIOLOGY, WASHINGTON, DC, US, vol. 173, no. 5, March 1991 (1991-03), pages 1677-1685, XP002119481 ISSN: 0021-9193 see whole doc. esp. discussion	
A	SORY M-P ET AL: "EXPRESSION OF THE EUKARYOTIC TRYPANOSOMA CRUZI CRA GENE IN YERSINIAENTEROCOLITICA AND INDUCTION OF AN IMMUNE RESPONSE AGAINST CRA IN MICE" INFECTION AND IMMUNITY, AMERICAN SOCIETY FOR MICROBIOLOGY. WASHINGTON, US, vol. 60, no. 9, 1 September 1992 (1992-09-01), pages 3830-3836, XP002070535 ISSN: 0019-9567 the whole document	
A	WO 89 10137 A (UNIV LOUVAIN) 2 November 1989 (1989-11-02) see whole doc. esp. claims	

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/EP 02/03372

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 32-43,45,52,54,55 , are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

nformation on patent family members

In tional Application No
PCT/EP 02/03372

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5965381 A	12-10-1999	AU 2850199 A	20-09-1999
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